ARS 45-5
May 1966

FACTORS INVOLVED IN HOST-AGENT RELATIONSHIPS

A symposium held at the dedication of
the National Animal Disease Laboratory,
Ames, Iowa, December 12 and 13, 1961

Agricultural Research Service

UNITED STATES DEPARTMENT OF AGRICULTURE

FOREWORD

The National Animal Disease Laboratory at Ames, Iowa, is the U.S. Department of Agriculture's research center for studying livestock diseases that occur in the United States. When the laboratory was dedicated by the Honorable Orville L. Freeman, Secretary of Agriculture, many distinguished guests participated in the ceremonies. During the 2 days preceding the dedication, a symposium for the scientific guests was held at Iowa State University. Prominent scientists and research workers from the United States and some from foreign countries participated in the symposium. The discussions are presented in this publication. Much of the material has been prepared from tape recordings made at the symposium.

Scientific research is a sampling procedure, a process by which broad conclusions and applications are drawn from experience with limited numbers. When animals are involved, it is important to recognize that all animals are not alike and no two of them are identical in all respects. Therefore, the scientist is warned to be exceedingly cautious not to generalize too much on experiences with limited numbers. This warning set the theme for the symposium and the various speakers presented evidence to substantiate its validity.

Editing of the symposium proceedings and arrangements for their publication were completed by the late Dr. William A. Hagan. Dr. Hagan was director of the National Animal Disease Laboratory from its inception until his death on February 1, 1963.

Howard W. Johnson, Director, Animal Disease and Parasite Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland

CONTENTS

Page

SECTION I--VARIATIONS IN EXPERIMENTAL ANIMALS:

Biochemical and Physiological Variations Within Groups of Supposedly Homogenous Experimental Animals, by ROGER J. WILLIAMS..... 1

Pathogen-free Animals as Experimental Hosts, by GEORGE A. YOUNG..... 12

SECTION II--FACTORS INFLUENCING HOST SUSCEPTIBILITY TO INFECTION:

Nutrition as a Factor in Disease, by ANDREW T. PHILLIPSON..... 23

The Role of Chemicals and Hormones in Influencing Host Susceptibility, by ALVIN S. LEVINE..... 37

The Importance of Scientific Research for Improving Livestock Production and Health in High Altitudes, by TEODORO RAMOS-SACO..... 50

SECTION III--METHODS OF STUDYING HOST REACTIONS TO INFECTION:

Histochemistry of Host Response to Infections, by MAX WACHSTEIN..... 59

Immunochemical Methods, by KEITH M. COWAN..... 83

Host Reaction to Disease: Application of Radionuclides, by LEO K. BUSTAD and R. O. McCLELLAN..... 94

SECTION IV--HOST REACTIONS TO INFECTION:

Some Methods for the Study of Morphological Cellular Changes Caused by Viruses, by HERMAN R. SEIBOLD..... 111

Relation of Some Serum Inhibitors to the Cell Receptors for Polyoma Viruses, by W. WILBUR ACKERMANN..... 120

Host Cell Interaction with the BAI Strain A Avian Tumor Virus, by HIDEO ISHIGURO, R. A. BONAR, URSULA HEINE, GUY de THÉ, DOROTHY BEARD, and JOSEPH W. BEARD..... 134

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SECTION I - VARIATIONS IN EXPERIMENTAL ANIMALS

Chester A. Manthei, D.V.M., assistant director for research, National Animal Disease Laboratory, presiding [1]

BIOCHEMICAL AND PHYSIOLOGICAL VARIATIONS WITHIN GROUPS OF SUPPOSEDLY HOMOGENOUS EXPERIMENTAL ANIMALS

Roger J. Williams, Ph.D. [2]

Variations such as I will speak of appear to be cruel blocks in the way of those who are trying to reduce biology to a science, yet they are a part of nature and there is no escape from them unless one deliberately closes his eyes. The recognition of such variations is, however, by no means futile from the scientific standpoint, because there are numerous problems which cannot by any means be solved unless such variations are taken into account.

I will admit that my fundamental interest is in a type of "experimental animal" that is particularly difficult to deal with. Sometimes it is said that human beings are poor experimental animals. This is true, but they are, from the human view at least, by far the most important "experimental animals" of all. My main discussion will, however, deal with experimental animals in the more usual meaning of the term-not, I hope, without taking an occasional sidelong glance in the direction of human beings.

1/ Papers with the following titles were also presented at the symposium but were not available for publication: The Significance of Histological Differences among Species in Relation to the Recognition and Interpretation of Lesions, by Thomas C. Jones, D.V.M., Angell Memorial Animal Hospital, Boston, Massachusetts; and the Anatomical, Histological and Physiological Characteristics of Germ-free Animals, by Richard A. Griesemer, D.V.M., School of Veterinary Medicine, Ohio State University, Columbus, Ohio.

The literature citations at the end of each symposium paper follow the order and content submitted by the author.

Dr. Manthei was appointed director of the laboratory, May 6, 1963.

2/ Clayton Foundation Biochemical Institute and Department of Chemistry, University of Texas, Austin, Texas.

One of the most thorough and impressive studies that bears upon my thesis is that of Wade Brown and coworkers (1) 3/ at the Rockefeller Institute in 1926. They dissected 645 well-nourished male adult rabbits such as were used for experimental purposes. They found the indicated ranges in the relative organ weights after correcting for the fact that the animals were not completely uniform in weight. As noted in table 1, the variations are unbelievably great. They average fourteenfold, and the median range is tenfold.

If one were to center his interest on the average rabbit-the one that is about average in every respect-a little calculation will reveal a serious dilemma. If we consider only those animals in the middle 50 percent of the complete range for each of the items, we find that when each measurement is considered in turn, one-half of the remaining population (the lower and upper quartiles) is excluded, and as a result it would require a population of 65,536 rabbits to yield one specimen that was in the middle group with respect to all 17 items. Surely such an extraordinary specimen could not be considered typical of the group. Even if we broadened our range to include animals in the middle 90 percent of the complete range for all 17 items, five-sixths of all the rabbits would be excluded. By no reasonable assumptions is it possible to arrive at a picture of an average (statistical) rabbit that may be used as a prototype; that is, representative of all the 645 male rabbits dissected.

One way out of this dilemma is to admonish ourselves thus: "Do not use mere 'rabbits' as experimental animals; instead, use more closely inbred specimens, which will be more uniform in character."

Among experimental animals, rats have been extremely popular, particularly for studying in the wide area of nutrition. Out of a desire for uniformity has been developed the albino rat, which is uniformly white-and it is sometimes supposed to be also uniform in other respects.

We studied the urinary excretion patterns of seven albino rats selected from our colony (2). These, so far as appearance and general breeding were concerned, were uniform and were getting precisely the same food and the same environmental treatment. Yet their patterns were highly non-uniform. The urinary phosphate excretion varied among these seven animals over about a twentyfold range; aspartic acid, sixtyfold; taurine more than fortyfold; and lysine, one hundred twentyfold! It is clear that the albino rat is, metabolically speaking, far from a uniform creature.

3/ Numbers in parentheses refer to Literature Cited at the end of this paper.

TABLE 1.--Range in relative organ weights of 645 rabbits

Organ	Grams per kilogram of net body weight		Ratio-maximum to minimum weight
	Minimum	Maximum	
Gastrointestinal mass-----	70.4	452.0	6
Heart----------	1.95	4.42	2
Liver----------	23.2	117.0	5
Kidneys----------	3.45	17.28	5
Spleen----------	.035	2.93	80
Thymus----------	.248	3.315	13
Testicles----------	.47	4.93	10
Brain----------	3.33	8.16	2.5
Thyroid----------	.048	1.23	25
Parathyroid----------	.001	.022	22
Hypophysis----------	.007	.035	5
Suprarenals----------	.080	.572	7
Pineal----------	.002	.025	12
Popliteal lymph nodes-----	.05	.382	8
Axillary lymph nodes------	.019	.24	13
Deep cervical lymph nodes-	.02	.295	15
Mesenteric lymph nodes----	.67	6.91	10

If albino or other rats are inbred for many generations by brother-sister mating, their excretion patterns show more uniformity than is exhibited by the group of animals cited above, yet the patterns are still far from uniform. Two rats in our study were actually from the same highly inbred strain, yet there were some striking differences. The phosphate excretion between these two animals varied fourfold; the glutamic and beta-amino isobutyric acid excretions varied twofold; lysine was "present" in the urine of one rat but not in the other; one unknown chromatographic spot was present in the urine of one but not in the other.

Our conclusions from these observations, which I admit are disturbing, are that no strain of rats, no matter how closely inbred, exhibits uniformity to such a degree that an average specimen can be selected and safely used in careful quantitative studies as representative of the group. Even if one were successful by some intricate breeding program in producing a strain of uniform experimental animals, the usefulness of such animals would be severly limited, because such a group could not be compared with any other group of animals unless it too had been produced by similar means.

The rats for which the urinary patterns were recorded were on the same animal-chow diet. While eating this same food, one rat, for example, excreted 60 times as much phosphate in the urine as did another of the same inbred strain.

Here is a point at which we may well take a glance in the direction of human beings. One of the reasons for animal investigations rests upon their application to human betterment. Surely, human beings are far from inbred, and conclusions from animal experiments arrived at by using homogeneous animals (if such existed) would be limited in value so far as human beings are concerned. The same principle applies, in fact, to farm animals and pets. One of the reasons for using experimental animals is to throw light on problems associated with farm animals and pets. If it were possible by some legerdemain to produce a group of uniform animals for experimentation, such animals would hardly be useful directly for solving problems related to farm animals and pets, which are generally speaking never as uniform as, for example, albino rats.

Realizing that the production of uniform animals for experimental purposes is not practical or even desirable, we have studied available strains and have sought to learn more about their individual variability and the basic causes and implications of it.

An experiment performed by my associate, Richard B. Pelton (6), illustrates how the internal biochemical individuality among inbred rats is reflected in their behavior. It involved 39 Holtzman rats, which were given a "cafeteria" selection of food. Each rat was provided with six dishes containing, respectively, (1) dried lean meat, (2) fresh carrots, (3) butter, (4) sugar, (5) salt mixture, and (6) fortified yeast. A record was kept for 17 days of the consumption of each item by each rat. At the same time that this experiment was being conducted, we gave the animals a choice of two beverages-water and 10-percent alcohol. The consumption of the alcohol, as well as the other articles, by each animal is summarized in table 2. It will be noted that the variations from animal to animal are great. The average spread of consumption among the items is tenfold with a median of fourfold.

Another series of experiments involved studying the tendencies of these and other animals to exercise. Rats given access to exercise wheels usually required a period of learning. They tended to spin the wheel more as they gained experience. Female rats exercised much more during the estrus cycle and, even excluding this period in general, they exercised more than did males. We have taken these facts into account in carrying out experiments.

Table 3 summarizes the test results on voluntary exercise obtained with 11 Holtzman male rats, 10 Sprague-Dawley females, each of which was tested on 4 to 6 days spaced in such a way as to avoid their estrus cycle, and 9 Wistar female rats.

In these three experiments, the tendency to exercise varied among the individual animals-sixfold among the Holtzman males, sixtyfold among the Sprague-Dawley females, and twenty-sixfold among the Wistar females. The variation for all groups was two hundredfold-a low of about 160 feet per day and a high of 6 miles per day. Averages for particular animals were those of four to seven trials, each lasting 24 hours.

The basic reasons for these wide variations are probably anatomical and hark back to the enormous differences in organ weights found in rabbits by Wade Brown (table 1). We do not have extensive data on the organ weights of inbred rats, but what we have indicates that the variation is substantial. Particularly pertinent and difficult to obtain would be the variation in the sizes and activities of the various endocrine glands and in the production of individual hormones. The differences in the tendency to exercise cannot be ascribed to something as simple as thyroid differences. Some rats have their exercise tendency elevated appreciably by thyroid administration, but the effect is not enough to transform a low exerciser into one with a high tendency to exercise. Some rats even have their tendency to exercise diminished by thyroid administration.

TABLE 2.--Food choices of 39 male Holtzman rats for a 17-day period

Rat	Percentage of dry ration consumed									
	Dried lean meat	Dried fresh carrots	Butter	Sugar	Salt mixture	Fortified yeast	10 percent alcohol	Total ration eaten (dry wt.)	Total weight gain	Ratio of weight gain to ration eaten
							ml. 100 rat/day	Grams	Grams	
A----	29.4	15.8	11.2	12.6	1.6	29.4	1.34	199.8	75	0.375
B----	32.1	13.5	15.2	4.9	1.5	32.8	1.52	196.9	81	.411
C----	47.6	9.8	12.3	11.9	1.0	17.4	1.00	209.4	99	.472
D----	47.0	20.6	15.6	10.5	2.1	4.2	2.72	187.1	75	.400
E----	32.6	32.5	16.8	13.0	1.4	3.7	1.85	195.0	62	.317
F----	40.9	20.6	17.9	13.4	2.3	4.9	8.20	152.0	62	.407
G----	17.5	27.6	12.6	37.8	1.8	2.7	2.97	153.9	34	.220
H----	47.7	20.9	9.1	9.6	1.8	10.9	6.65	181.3	76	.419
I----	28.0	15.9	6.9	5.5	.6	43.1	.90	233.8	77	.329
J----	45.4	17.1	4.2	7.4	1.3	24.6	2.38	208.4	79	.379
K----	36.8	29.1	7.4	21.1	1.2	4.4	.96	188.4	64	.339
L----	39.9	10.4	15.7	7.5	1.8	24.7	.72	183.4	83	.452
M----	29.2	14.4	7.9	20.0	1.1	27.4	.89	222.2	77	.346
N----	41.1	19.5	12.5	15.8	1.2	9.9	1.95	190.7	81	.424
O----	36.8	11.5	7.4	9.9	1.9	32.5	4.17	232.3	84	.361
P----	48.0	12.0	6.3	6.9	1.6	25.2	.92	208.0	84	.403
Q----	34.8	29.9	11.1	8.7	1.1	14.4	1.57	200.8	78	.388
R----	35.4	17.9	12.7	9.4	1.5	23.1	1.09	223.1	82	.367
S----	19.8	20.7	15.4	28.6	1.2	14.3	2.16	159.0	51	.320
T----	43.8	17.6	9.1	23.8	1.5	4.2	3.07	196.9	88	.446
U----	44.5	15.7	8.6	14.5	1.4	15.3	3.46	211.9	90	.424
V----	49.7	16.5	17.7	12.1	1.3	2.7	1.48	172.6	85	.492
W----	58.6	17.3	17.8	3.5	1.8	1.0	4.50	184.5	86	.466
X----	53.6	8.2	7.4	11.7	1.9	17.2	1.92	182.9	77	.420
Y----	44.5	21.9	15.2	11.3	2.2	4.9	1.04	171.2	63	.367
Z----	37.2	14.5	6.4	5.3	.9	35.7	1.27	170.3	69	.403
AA----	48.8	17.5	11.8	12.0	1.8	8.1	1.05	197.1	84	.426
AB----	52.1	13.4	12.5	16.5	1.8	3.7	3.24	161.4	58	.359
AC----	15.7	21.9	12.4	2.4	1.1	46.5	1.34	207.4	65	.313
AD----	25.8	18.6	4.1	41.8	1.1	8.6	1.90	191.0	63	.329
AE----	41.2	21.1	2.6	27.5	1.2	6.4	1.73	227.0	75	.330
AF----	50.7	17.6	8.6	17.6	1.9	3.6	1.65	189.4	72	.380
AG----	43.5	16.1	12.5	8.6	1.1	18.2	1.97	145.7	61	.418
AH----	55.3	10.8	14.8	11.0	1.8	6.3	5.25	148.6	62	.417
AJ----	28.7	11.8	6.1	17.4	1.6	34.4	.78	227.6	69	.303
AK----	56.5	18.1	12.2	6.4	1.8	5.0	3.17	159.5	61	.382
AL----	37.1	20.7	19.2	17.9	1.4	3.7	1.99	165.3	68	.411
AM----	41.6	20.1	14.0	5.0	2.1	17.2	1.95	200.4	74	.369
AN----	19.1	13.1	19.2	2.6	1.3	44.7	1.28	168.7	51	.302
Mean--	39.4	17.9	11.5	13.4	1.5	16.3	1.96	189.9	72	.382
Extreme-	15.7-58.6	8.2-32.5	2.6-19.2	2.4-41.8	.6-2.3	1.0-46.5	.72-8.2	145.7-233.8	34-99	.220-.492
Ratio of high to low---	4	4	7	17	4	46	11	2	3	2

TABLE 3.--Voluntary exercise of three groups of rats

Revolutions per 24 hours on 14-inch wheel

Rat	Test number-- 1	2	3	4	5	6	7	Mean for all tests
HOLTZMAN MALES								
V----	1,553	1/ 695	695	1/ 1,391	1,391	1,346	1,028	1,157
W----	783	1/ 765	765	1/ 1,428	1,428	1,503	1,129	1,114
AA---	1,160	1/ 1,288	1,288	1/ 2,028	2,028	2,152	2,304	1,750
AC---	1,746	1/ 1,852	1,852	1/ 2,629	2,629	3,859	4,480	2,721
AD---	1,001	1/ 1,199	1,199	1/ 1,334	1,334	1,414	1,127	1,230
AE---	1,531	1,449	2,295	2,245	3,439	2,624	2,054	2,234
AF---	1,196	1,888	794	3,370	1,418	3,175	1,839	1,954
AG---	628	510	601	1,039	574	546	294	599
AJ---	1,091	1,532	1,308	2,690	4,018	3,480	2,740	2,408
AL---	731	626	559	381	278	307	323	458
AM---	1,439	2,025	1,475	2,157	1,911	1,635	1,308	1,707
SPRAGUE-DAWLEY FEMALES								
11---	92	209	568	536	1,076	611	---	515
12---	57	68	1/ 127	99	---	---	---	88
13---	182	315	1/ 662	662	---	---	---	455
14---	15	244	1/ 147	147	---	---	---	138
15---	36	22	1/ 57	57	---	---	---	43
16---	1,134	1,409	1/ 1,692	1,652	---	---	---	1,462
17---	1,106	1,033	1/ 1,627	1,627	---	---	---	1,348
18---	891	442	1/ 2,459	2,459	---	---	---	1,563
20---	1,375	1,681	3,354	3,958	---	---	---	2,592
21---	1,901	1,512	2,822	766	---	---	---	1,750
WISTAR FEMALES								
1----	412	76	257	359	712	188	---	334
2----	2,623	2,443	4,452	1,947	2,055	1,982	---	2,584
3----	1,400	1,301	2,803	2,571	4,244	8,321	---	3,440
4----	2,086	4,881	8,584	10,758	11,970	13,386	---	8,611
5----	1,004	1,093	2,611	2,415	3,860	3,815	---	2,466
6----	2,523	2,177	3,172	4,616	6,554	3,732	---	3,796
7----	2,800	2,174	4,309	8,387	7,881	4,004	---	4,926
9----	1,010	438	997	1,445	1,478	726	---	1,016
10---	1,756	2,612	2,357	1,783	3,752	827	---	2,181

1/ Revolutions recorded after 48 hours on exercise wheel. This value and the value for following day are for half of revolutions for 48-hour period.

Experiments conducted by my associate, Frank L. Siegel (3) 4/ show clearly that wide variation within inbred strains is not limited to rats. In figure 1 we have diagrammed the susceptibility to intoxication of four male baby chicks, all of them the DeKalb 101 strain and obtained from the same hatchery at the same time. The susceptibility rating was based upon the observations that the first signs of intoxication were lowering of the head and an appearance of listlessness. The second sign was staggering; the third, was taking a squatting position and remaining immobile; the fourth, involved toppling over and lying on the side or back; the fifth, appeared when chicks failed to respond to any stimulus such as nudging.

It will be noted that some chicks responded about the same to repeated tests (whether high or low in susceptibility), other chicks were more variable. When tested individually in small groups, chicks of the same strain also varied over about a tenfold range in their voluntary consumption of alcohol when given a choice between 4 percent alcohol and water. Their appetites were likewise impaired to greatly different degrees. When chicks were each given a carefully weight-graded dose of alcohol, some were able to eat a normal amount and to grow and thrive. At the other extreme, some chicks lost their appetites almost completely and died of inanition within a week.

It may be mentioned that these experiments were not done because of a primary interest in the effects of alcohol on baby chicks. Our primary interest was that human beings respond to alcohol in many diverse ways. This variability in response, we have reason to believe, has a biological basis.

The subject matter of my discussion has many and manifold implications, some of which are discussed in my book, "Biochemical Individuality" (4). Certainly noteworthy are the implications for nutritional research. It seems obvious that if biochemical individuality exists to the degree that it appears to, the nutrition of individual animals and of individual human beings is worthy of more attention than it has received (5).

One of the important applications of the idea of individuality has to do with susceptibility to disease. This, in human beings, is an individual matter and is the basis for a new branch of medical science, which we have called "propetology" (7). The same idea can be carried over and indeed must be carried over to veterinary medicine. Because of their biological individuality, individual animals like individual human beings must have their own tendencies toward specific diseases.

4/ Siegel, Frank Leonard, 1960. Individuality in Baby Chicks as Related to Their Responses to Alcohol. Ph.D. dissertation, University of Texas, Austin, Texas. (U. S. Library of Congress microfilm no. 61-1389).

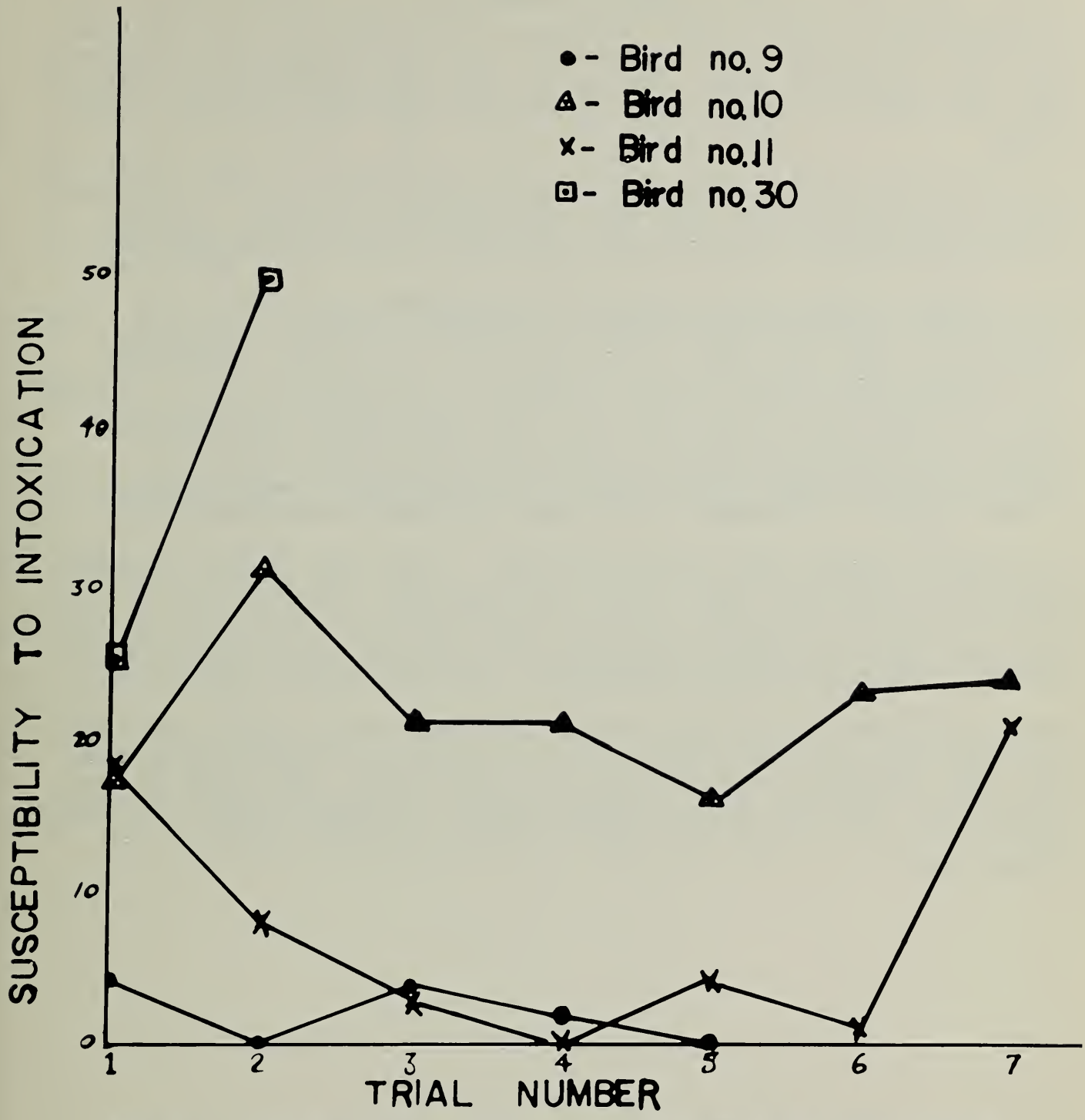

Figure 1.--Susceptibility to intoxication of four male baby chicks.

I hope I have given enough examples to make each of my hearers dubious, or at least mildly critical, about anything of a detailed nature that is said about the rat, the baby chick, the guinea pig, the dog, the cow, the horse, the hog, or the human being. What we can say conservatively on a scientific basis about each of these is often obvious and elementary and hardly worth saying.

In answer to a question on whether the rats used in the exercise experiments were of the same age--yes, they were, when tabulated together. We have worked with animals of various ages, most often with young weanling rats.

How do these findings affect the validity of numbers to be chosen for statistically sound results? If one is trying to use animals to study something to which they show a wide variability in response, then relatively large numbers must be used. This came up some years ago in connection with the biological determination of vitamin A. Because of the wide variability in the needs for rats for this particular vitamin, relatively uniform and responsive rats must be bred for the specific purpose of vitamin A determination. Then, when the determination is made, one has to average together 10 or maybe 20 rats before getting a dependable value.

One question touches upon a very sore point. I feel with respect to human beings that statistical "man" is not very important. The only kind of people that exist are individual ones. With regard to the statistical "rat", I also am highly indifferent. This doesn't mean that I am antistatistics. Actually, I am very much for statistics. I am only against careless use.

When one parameter is involved, we have averages, medians, modes, standard deviations, and the like. However, when one thinks of a whole entity with many, many parameters, such as a rat or a human being, these terms are not appropriate. There are men of average height, of average weight, of average length of foot or of nose, but there is--using statistics correctly--no average man.

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PATHOGEN-FREE ANIMALS AS EXPERIMENTAL HOSTS

George A. Young, D. V. M. [1]

My intent is to discuss pathogen-free animals in general. [2] The term "pathogen free" can apply to calves or lambs as well as pigs. However, I will primarily discuss pigs, because they happen to be the experimental host with which our particular laboratory has had the most experience.

The conventional pig has two drawbacks as an experimental host. One of these is that he shares an environment with his dam, so that the diseases that are common to her environment may be transmitted to the newborn pig. An experimentally induced disease is difficult or impossible to interpret when superimposed upon a naturally occurring disease, no matter how mild the natural disease.

The second problem involved in using the conventional pig as an experimental host is that very soon after dropping from the mother, the little pig suckles his dam. The colostral milk ingested at this time may contain antibodies to a number of pathogenic agents. All the diseases the dam has had throughout her lifetime are portrayed as immune globulins in her milk. Transmission of experimental disease to pigs that have nursed dams that previously experienced those diseases is very difficult. Even when experimental disease is established, it may be modified markedly because of partial passive immunity acquired from colostrum.

Nature has provided a means for us to acquire excellent experimental hosts in what is defined as the pathogen-free animal. The fertilized ovum develops into the germ disc and thence rapidly into an embryo. The fetal membranes, five layers in all, envelop the embryo between 15 to 25 days and serve as a barrier to bacteria or viruses that may be harbored by the dam. Some viruses at this very early stage may produce damage (21), but as the placental tissues become completed, it is either impossible or at least not probable that bacterial or viral agents will invade the developing embryo. Such an embryo develops into a fetus, and, when it persists until term as a normal entity, it is considered to be germ free.

1/ Department of Veterinary Science, University of Nebraska, Lincoln.

2/ The original presentation was illustrated by 80 visual slides that are not available for this publication. Literature citations are provided in the list at the end of this paper. The numbers in parentheses refer to Literature Cited.

The work we undertook in 1949 entailed obtaining a desirable type of animal for experimental purposes, that is, one that would not come in contact with the mother's environment and would also not suckle the dam and thus have the confusing aspects of colostral antibodies. We began by catching pigs in sterile canvas bags (18). This was a partially successful technique if there was no contamination during the passage through the birth canal or introduction of flatus or fecal material into the canvas bag. It was possible to obtain a pathogen-free pig under ideal conditions. To overcome the limitations of the sterile-bag technique, we developed a method of delivery of pathogen-free pigs by hysterectomy (20). This is a 5-day, 40-hour week procedure as far as we are concerned, because we can take little pigs from the sow at our convenience in 110 to 112 days after breeding. An incision is made on the midline and the uterus is dropped out and passed through an antiseptic lock into a hood, which has been sterilized previously (9). After the animals are torn out of the uterus, they are transported to the laboratory in a covered case, which is aerated through a filter medium. The case is also covered by a sterile canvas bag.

The animal attendant who handles the pigs at the laboratory wears clean coveralls, face mask, and boots. He steps into an antiseptic foot bath when entering a room, which has been gassed previously with formaldehyde, and places each little pig into a sterile modified Horsfall-Bauer isolation unit (19). The isolation unit is sealed so that the air coming in is microfiltered from the room and is exhausted through another microfilter into a common exhaust stack. The little pigs are fed sterile cow's milk from pans for the first day or two. After the first 2 days, the pigs receive ordinary pasteurized milk from which they pick up a harmless bacterial flora. This is the reason that we refer to these animals as pathogen free rather than germ free. A larger unit may be used for older pigs that are to be held in isolation for several weeks (10). We can conduct isolation experiments in this type unit until the pig weighs about 50 to 60 pounds.

Some consideration must be given to classification and terminology used in this general area of research. Common classes are germ-free, pathogen-free, and conventional animals. Germ free denotes absence of all microbes and is an absolute condition. I think it is very important that we study some of our diseases in previously germ-free animals. This is very difficult to do. It is very easy to have breaks in the procedure and contaminate the experimental host with unwanted bacteria or viruses. The term that is being used commonly by some of the laboratories is gnotobiote or "known-life." Subdivisions are alpha, beta, and gamma gnotobiotes. Alpha gnotobiotes are those animals used in reproductive studies. The gamma gnotobiote is the exact counterpart of the germ-free animal. The beta gnotobiote indicates a mono or known contaminated animal. I prefer to use pathogen free because actually it denotes the same thing in general as the beta gnotobiote, in that you attempt to define the background that these animals have. We are

mostly concerned with the pathogens that would affect experimental hosts as far as clinical disease is concerned. The term specific pathogen free (SPF) is an applied farm-level type of disease control. The conventional animal is one that has an undefined and unknown microbial background.

The newborn pathogen-free pig has some characteristics just a little different from those of his naturally farrowed counterpart. Upon nursing his mother, the naturally farrowed pig has a protein level that is slightly higher than that of the hysterectomy-derived pig, which has as its diet cow's milk fortified with a whole egg. The naturally born pig absorbs globulins rapidly. At the end of the first day, he has absorbed a large proportion of his total protein, which contains gamma globulins. The striking difference between the naturally farrowed pig and the pig that is farrowed by hysterectomy as shown by electrophoresis is indicated in gamma globulin levels. Electrophoretic patterns indicate that the hysterectomy pig has small amounts of globulin at birth. He does develop globulins slowly. In this pathogen-free state, you must remember that there are bacteria in the intestine and some globulin may result in acceptance of an adjustment to those bacteria in the intestinal tract.

Research on the fundamentals of the absorption of gamma globulin has disclosed some interesting facts. The intestinal mucosa of the little pig has demonstrable absorption of globulins within minutes after birth and feedings (3). Globulins were tagged with fluorescein and observed in tissue sections under ultraviolet light. Globulins were not absorbed when they were succeeded by actual feeding of milk that contained no globulins. A 4-day-old pig that was starved and then fed globulins was still capable of absorbing these globulins, whereas almost immediately, after a feeding with protein, absorption was blocked. Little pigs receiving injections of globulins intraperitoneally continue to absorb globulins. Sections of intestine examined 1 hour, 3 hours, 5 hours, and 7 hours after giving the fluorescence-tagged globulin indicate the rapidity of absorption. Absorption begins within a few minutes and continues for about 6 hours (17). Heterologous as well as homologous globulins are absorbed by the little pig. Studies have been made with globulins from human, bovine, and equine sources. All globulins are absorbed similarly. We thought once the absorption of antibody was around the cell to the base, but we now know that the cells engulf the globulins and pass them through the cell with a more or less pulsating action to the base of the cell and then inject them into the blood stream.

Some of the other fundamental studies that have been possible with this type of experimental animal include those in basic immunity. Serum block has been demonstrated in a number of animals. It has been shown in dogs, swine, and cattle that there is a block in development of active immunity by antigenic stimulus when colostral antibodies are present.

Colostrum, which does not contain specific antibodies, may act as a base to build new antibody. The little pig that has not had colostrum is a poor producer of antibody with Brucella antigen as the stimulus. However, viral antigens, that is, living antigens, given to animals at this stage do stimulate development of permanent immunity.

Age does affect the production of immunity. A 3-week-old pig has a very modest response to an antigen; whereas, when the first experience to the same antigen is at 6 weeks, there is a marked and relatively rapid response (2).

A modern trend in animal disease research is to use tissue-culture cell systems as experimental hosts. It must be emphasized that within the study of animal diseases, we must not lose sight of the use of the total host. These experiments illustrate the reason for concern in this area. Beran's enterovirus is cytopathogenic for swine kidney cells. When this virus is given to a pathogen-free pig, however, there is no clinical sign of disease. The virus attacks the cells of the intestinal tract, multiplies, and persists for 4 to 5 days. You can isolate the virus from this clinically normal pig and put it back in tissue culture. Immunity also develops to this virus and the little pig produces neutralizing antibodies with no evidence of clinical disease (1). In tissue-culture studies, this swine enterovirus can be stained with acridine orange and observed under fluorescent light. The developing colony can be seen in cells as an ribonucleic acid, or RNA virus. Development of the viral colonies is progressive.

By contrast, the appearance of a normal tissue-culture cell layer is maintained after inoculation with transmissible gastroenteritis virus of swine (TGE). This is an enteric virus, but it has opposing characteristics to the enterovirus reported by Beran and others (1), in that it destroys the pig. TGE is a very lethal agent as far as pigs under 1 week of age are concerned. Mortalities approaching 100 percent with profuse diarrhea and vomiting are characteristic of TGE. On placement of this virus in tissue-culture cells, there is no gross evidence that it has multiplied. However, by using acridine orange dye and the fluorescent light, we do find development of this particular virus in the tissue-culture cells but at an extremely slow rate.

Thus, contrasting viruses are defined by use of two host systems. One (TGE) is pathogenic for pigs, but did not produce cytopathogenic effects in tissue culture. The other (Beran's) is cytopathogenic for tissue-culture cells, but not pathogenic for pigs by any clinical signs. This demonstrates well that we must continue to use a total host in experimental work as an adjunct to tissue-culture systems.

Another excellent use of pathogen-free, colostrum-deprived pigs as experimental hosts is for studies of respiratory infections. Any animal that comes into a natural environment immediately starts to filter out the agents that are airborne within that environment by means of cilia in the upper respiratory tract. Many respiratory viruses are problems as far as animals are concerned. The pathogen-free animal makes an excellent host, because at birth its lung is free of any abnormality. Most of us have been taught that an atelectatic lung is normal for the newborn pig. This is not true. The pigs that are born by hysterectomy have lungs that do not have any atelectatic areas in them. The histology is that classically described in the textbooks. This is a rarity in a conventional animal.

One of the classics as far as respiratory infections are concerned in swine is the disease swine influenza, described by R. E. Shope many years ago (6,7). The bacterium Hemophilus influenzae suis does not alone cause disease. The virus itself causes what he called filtrates disease. The combination of the two produces a clinical disease, which is referred to as swine influenza. This is a clinical entity much more severe than the filtrates disease and certainly the bacterial disease, which in the pathogen-free host does not establish itself. The gross lesion of the filtrates disease with swine influenza virus resembles atelectasis in appearance. The histopathologic reaction is characteristic. Cells proliferate along the alveolar walls as the animal recovers, and much cellular debris is in the bronchioles.

Another respiratory disease in pigs that has a very high incidence, possibly 40 percent, may grossly look like swine influenza (16). Atelectatic areas in the apical and cardiac lobes histopathologically are quite different from those in swine influenza. Perivascular and peribronchial cuffing with round cells is the characteristic reaction of virus pneumonia of pigs (VPP) in these animals. The same type of reaction occurs in conventional animals. Quite often VPP is complicated with bacterial infections.

Some parasitic infections must be classed in a respiratory category. Migrating larvae of Ascaris suum begin as embryonated eggs, which are swallowed by the pig. They hatch, then pass through the stomach wall, liver, and lung and return to the alimentary canal and establish housekeeping there. Development of vermifuges has been directed toward elimination of this intestinal state. But this is not the part of this parasite's life cycle that is troublesome so far as we are concerned. We are more concerned about its migration through the lung and some of the things it does there. One of the things associated with this larval migration is the intensification of the effects of respiratory viruses by the simultaneous presence of the ascarid larvae and the virus itself. The lesion produced by VPP virus is relatively simple. The accentuated lesion produced by introduction of the VPP virus at the time the ascarid larvae are migrating through the lung is essentially tenfold that of the uncomplicated disease. The intensity of disease clinically is also increased (8).

We previously discussed Beran's enterovirus, which usually produces no lesion. However, Beran's enterovirus given intranasally at the time migrating ascarid larvae are going through the lung sets up an unusual situation in which enterovirus causes a virus infection of lung tissue with a demonstrable lesion.

One of the other parasites that we are concerned about is the swine lungworm. This parasite intensifies both swine influenza and virus pig pneumonia when the parasite is in the lung at the time the virus is also present. In simple ascariasis, the pig eats the ascarid eggs, and in the migration through the liver and lung, pinpoint hemorrhages are evident in the lung. The agent causing virus pig pneumonia is present during this migration, and the ascarids precipitate an intense pneumonia, whereas the virus alone causes only a mild pneumonia. There is a similar intensification of swine influenza by migrating ascarid larvae (11).

In the classical experiments of Shope (6,7), the swine lungworm was shown to be an intermediate host for swine influenza virus. We have confirmed Dr. Shope's work in our laboratories in the past 2 years, and we concur with his concepts entirely (5). The pigs eat the earthworms, which contain the lungworm larvae, which in turn contain swine influenza virus. A predisposing situation occurs when the lungworms and virus are present in the lungs of the pig, but the virus is in a masked state. The migrating ascarids cause release of masked virus to cause an influenza infection. Virus then may be spread to other animals within a natural environment. Ascarids are present on nearly all our midwestern farms. Thus, an accentuation of the disease from the migrating ascarid larvae would be expected to occur frequently.

There is considerable versatility with a pathogen-free host. It gives you an opportunity to study a number of things and has less variability than you might expect in the conventional animal. We have been interested in amino acid amidase activity as it relates to disease in tissue culture and the total host. Our main interest has been in leucinamidase because it is the most active of five enzymes we have studied in a disease situation. Substrates are hydrolyzed to cleave off free ammonia, which is evaluated quantitatively by colorimetric analysis (4). Infected lungworm larvae, which were infected with influenza virus, were fed to pigs. When ascarid larvae were fed and migrated through the host, the influenza virus was released to cause an active influenza infection. Activity of leucinamidase increased markedly. The normal animal served as a control with no increased enzyme activity. A mild reaction to the migrating ascarids was also noted (22).

We have used a technique to isolate some viral agents which we might not have isolated otherwise. Ordinarily we necropsy an animal, take blood or tissues, grind these up, and put them back into a host to attempt an experimental transmission. We may miss the active virus by these methods,

but by actual contact between a pathogen-free animal and a conventional animal, viruses may be transferred. There is an increased opportunity to effect an experimental transmission. The pathogen-free pigs are exposed and then put back in individual isolation for observation.

Four diseases I will mention briefly were originally uncovered by controlled exposure of pathogen-free pigs to sick conventional pigs. One disease we thought was swine dysentery because of an intense enteritis was caused by hog cholera virus. It was extremely virulent and caused typical perivascular cuffing with a marked karyorrhexis. After we made several passages of this virus in pathogen-free pigs, the virus became less virulent, and histopathologic reactions were less severe.

Histopathologically we could not be certain that this was hog cholera and still had to go back to tests with immune pigs that would resist challenge inoculation with a known virulent hog cholera virus. The reaction to virulent hog cholera is very little different in the pathogen-free, colostrum-deprived pig than you would expect in the conventional animal.

Another disease that we have been studying is one that we call Nebraska University disease (NUD) (12). This disease closely resembles field cases of hog cholera and swine erysipelas in some ways.

Another disease with similar characteristics is one we named edema disease (13) because of associated edema in various parts of the body. Edema is rather variable in this disease, which is also like hog cholera in many respects. The typically petechiated kidneys would confuse a clinician and make him think in terms of hog cholera. We can differentiate between hog cholera and edema disease by serological means, whereas the pathologist may have considerable difficulty in telling them apart. If the disease agent passes through a 0.5 Seitz filter, it is a filter-passing agent. Serum neutralization antibodies are specific (15). Meningitis is very common in both NUD and edema disease and is represented more or less by inflitration of round cells. The gall bladder may show thickening of the wall in edema disease, which presents an opportunity for confusion with African swine fever, especially when the clinical characteristics of edema disease that resemble hog cholera are manifested. There is no perivascular cuffing, but there is much edema around the vessels, which are permeable and permit fluids to escape into the surrounding tissue. There is a very rapid rise in leucinamidase activity. Intraperitoneal injection with the edema disease agent results in death within a few hours, with a marked increase in serum leucinamidase.

Some of our people in the study of latency of viruses in pigs have first given pigs the NUD agent followed by a modified, living, attenuated hog cholera virus. A third agent, that of edema disease, caused death within

24 hours. A large number of mitotic figures within the liver cells suggest that the liver was attempting to repair itself under the stress of these particular agents in rapid succession.

Another disease we studied is one that we first identified under some unusual circumstances in 1954. We found it as a contaminant in a biological product that was about to go on the market. This happened to be at the time of vesicular exanthema (VE), and we were concerned at the time that it might be the VE agent. After fourth passage by contact, the original agent caused erosion of the surface of the tongue and also some lesions on the snout. The last few months we have again picked up in the field what we think is the same agent. It causes an experimental disease many of the practitioners would diagnose as greasy pig disease or exudative epidermitis. Our first contact animals yielded a filterable agent and also a staphylococcus. By scratching the skin with material that contained the bacterium and the filterable agent together, a severe local lesion was produced followed by systemic infection. The filterable agent introduced alone caused a mild disease. Filtrates that passed an 0.5 Seitz filter caused mild lesions, which also involved the tongue. Mild lesions also developed from 0.1 Seitz filterates and indicated a very small agent (14).

I would like to emphasize that the use of pathogen-free animals as experimental hosts in animal-disease studies has real significance today. We are dedicating here a $16-million physical plant, which is going to be staffed with very capable people. The type of work to be done will depend largely on the quality of the experimental animals they use. It would seem pointless to have everything else under control and yet use animals of questionable quality. Perhaps this discussion of pathogen-free, colostrum-deprived pigs will stimulate the use of similar animals in the new National Animal Disease Laboratory.

* * * * *

Question: Did you say that you had found antibody absorption in the gut of these pigs in 4 days?

Answer: That is true. The condition of absorption was previous starvation of the animal for 4 days. Now as to the thing that triggers this inability to absorb--animals that ingest other proteins before attempted absorption will not pass globulins. If you starve the little pig up to 4 days, he will absorb the antibody or globulin.

Question: Did you find it in the serum?

Answer: Yes. Now, the question arises as to what age you could use these pigs for evaluating hog cholera. I would say that by 7 days you have an ideal host. These little pigs have learned to eat well, and they are quite vigorous. Their response to viral inoculations at 7 days is very similar to what it would be at any age. I might add that the pathogen-free pig is quite resistant to erysipelas bacteria. In fact, it is difficult to make these pigs take an experimental infection.

Question: Have you done neutralization tests with the greasy pig agent?

Answer: We have not and we should. I've discussed with people in your group the possibility of getting some serum for neutralizing tests and doing the experimentation here or in Nebraska.

Question: Is there any factor that you can control in the earliest ages of these little pigs to induce an earlier antibody response than 6 weeks, for example?

Answer: From our experience, you might give pigs small doses of antigen as more or less precursors, followed by stimulatory antigen, and expect a response. I think that they'll start producing some type of immunity from 3 days on with viable antigens.

Question: What if you neutralize the colostrum antibodies first? Would that help speed up the immunity?

Answer: I cannot say.

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SECTION II - FACTORS INFLUENCING HOST SUSCEPTIBILITY TO INFECTION

C. D. Van Houweling, D.V.M., assistant director for regulatory activities, National Animal Disease Laboratory, presiding 1/

NUTRITION AS A FACTOR IN DISEASE

Andrew T. Phillipson, M.R.C.V.S., Ph.D. 2/

Evidence shows that, under closely controlled experimental conditions, diet does alter the resistance to infection of experimental animals such as mice. The subject is a perfectly respectable matter for scientific inquiry and, as mentioned by one worker (13) 3/, there can be no grounds for the comment, "Is there really anything in this story about diet and infection?"

As long ago as 1924, (26) workers showed that newborn mice reared on one of two diets had a marked difference in susceptibility to experimental stomach-tube infection with one of the mouse-typhoid organisms, Salmonella typhimurium. The difference was obvious and repeatable. On the diet favorable to resistance, only 16 percent of 69 mice died, whereas on the diet less favorable to resistance, 78 percent of 56 mice died. The favorable diet consisted of 67.5 percent whole wheat, 15 percent casein, 10 percent milk powder, 1 percent salt, 1 percent calcium carbonate, and 5 percent butterfat. The less favorable diet consisted of baker's bread soaked in pasteurized milk, supplemented twice weekly with an oatmeal and buckwheat mixture and once weekly by feeding of dog biscuit.

1/ A paper with the following title was also presented at the symposium but was not available for publication: Influence of the Host's Genotype on Susceptibility to Infection, by Clyde Stormont, Ph.D., School of Veterinary Medicine, University of California, Davis.

2/ The Rowett Research Institute, Bucksburn, Aberdeenshire, Scotland.

3/ Numbers in parentheses refer to Literature Cited at the end of this paper.

There are many such experiments described in the literature. Previous work (23, 24) showed that mice maintained on a diet containing 92 percent of oatmeal were more susceptible to Salmonella than mice maintained on a diet in which oatmeal was reduced to 40 percent of the ration and the balance was replaced by dried skim milk, dextrin, and coconut oil. Mice on the poor oatmeal diet not only were more susceptible to Salmonella but also had a poorer breeding performance than those on the better diet. Other workers (21) compared a diet of whole wheat, dried milk, and salt with a synthetic diet and found that the whole wheat diet conferred a striking resistance to Salmonella infection compared with the synthetic diet. Replacing wheat by either dried cooked potato, whole corn, whole rye, whole oats, or whole rice showed that there was not much difference in the capacity of mice maintained on these diets to resist Salmonella, but undersized mice raised on oats and potato were very susceptible. Those reared on rye, rice, or corn were not susceptible.

The question to ask is not whether there is anything in this story about diet and infection, but "How does diet influence resistance to infection?"

Effect of Protein on Susceptibility to Infection

There is considerable evidence from carefully controlled experiments on mice that the quantity and nature of the protein in the food affect the susceptibility of mice to experimentally induced infections. A careful study (7) using diets otherwise adequate, compared the susceptibility of mice to injected standard doses of Staphylococcus aureus, Klebsiella pneumoniae type C, Mycobacterium tuberculosis var. bovis or Mycobacterium fortuitum when maintained on diets containing 20 percent casein, 8 percent casein, or 8 percent casein reinforced with 12 percent of an amino acid mixture containing acids essential for plasma globulin formation. In all such experiments, variation in the degree of response from experiment to experiment is usual, but the picture that emerged was that mice maintained on the 20 percent casein diet were more resistant than those maintained on the 8 percent casein diet. Supplementation of the 8 percent casein diet with amino acids restored the resistance to approximately the same as that of mice given the 20 percent casein diet. In this series of experiments it was found that the differences disappeared with time and that when mice were tested 14 days after being placed on these diets differences between diets occurred, but these differences disappeared when the mice were tested after 37 days on the experimental rations.

Further work showed that the kind of protein was also involved, for mice fed on the diet containing 20 percent wheat gluten--a protein with a low lysine content--were less resistant to Staph. aureus and to bovine tuberculosis than mice fed on the 20 percent casein diet. Wheat gluten did not produce such good weight increases in mice as the same quantity of casein, but wheat gluten supplemented with lysine was equal to casein in sustaining weight increases but did not increase the resistance of the mice to infection with K. pneumoniae type C, although resistance to Myco. tuberculosis var. bovis was improved by the addition of lysine to the wheat diet.

Mice on diets containing corn as a sole source of food or a proprietary pelleted diet containing 24 percent crude protein were also more susceptible to tuberculosis or to K. pneumoniae than mice receiving the 20 percent casein diet. Similarly alpha-protein of soybean was found to be less effective than casein in conferring resistance to Staph. aureus, although the animals had to be maintained for 20 days on this diet before the difference was appreciable. Diets containing mixed vegetable protein with an amino acid composition similar to that of casein, however, produced about the same degree of resistance as the 20 percent casein ration.

The route by which infections were given enhanced or depressed the rate at which resistance was overcome. The intraperitoneal route produced a more rapid response with a much smaller dose than the intravenous route, whereas nasal infection by airborne spray producers had a far slower response. The general conclusion (19) was that the composition of the amino acid mixture fed was responsible for the differences exerted by the proteins tested. All diets were supplemented to insure proper balance.

These experiments show that the quantity and quality of the protein of the food affected not only the speed with which mice died after the particular pathogens were injected but also the numbers of mice that died. A study of the figures, however, suggests that the effect of the protein in the ration was only one among various factors concerned, for the results from experiment to experiment were not entirely consistent as regards the severity of the response obtained, and the differences between diets were more marked on some occasions than on others. It should also be noted that the synthetic diet containing 8 percent casein was inadequate in its amino acid contribution to support more than a reduced rate of weight increase, whereas a wheat gluten diet sometimes did not allow a weight increase at all.

Effect of Vitamins on Resistance to Infection

The effect of vitamins on resistance to infection has been explored in considerable detail. In spite of the hopes centered on vitamin A, owing to its effects on epithelial tissues, any effect it may have seems to be entirely subsidiary to the anatomical changes induced locally in epithelial tissues, rather than to a specific effect on the resistance of the animal generally. There is no convincing evidence of a general effect on resistance to infection. As regards vitamin C, I can do no better than quote (20) to the effect that it prevents scurvy, but that is all.

Concerning deficiencies of the various B vitamins, there is a solid body of evidence to show that lack of these factors does increase susceptibility to bacterial infections, and that it does so presumably because of its effect on intermediary metabolism and especially on the synthetic processes concerned in the synthesis of globulins. The inanition that occurs in all forms of deficiency is unlikely in itself to lower the resistance of test laboratory animals to test pathogens for the specific vitamin B deficiencies, of which thiamine, niacin, and pyridoxine seem to be the most important.

Extensive investigations of the possible immunological mechanisms that may be affected in rats due to individual vitamin B deficiencies have been made (27, 28, 29). The most tangible change found was that complement activity and the rate at which cellular migration occurred to sites of inflammation seemed to be reduced. It is seldom that vitamin B deficiencies occur singly in present-day animal nutrition, and they are more likely to occur as an accompaniment of general malnutrition. Their effects seem to be due to a depression in the metabolism of tissues generally rather than to any specific effect on any specific immunological mechanism. It can only be assumed that tissues in a lowered state of metabolism are less resistant to bacterial toxins or surface toxins than those in a normal state of metabolism. It is perhaps relevant here to recall Pasteur's experiment, which showed that lowering the temperature of the chicken body made it more susceptible to anthrax.

The situation with virus infections seems to be that animals in a poor state of nutrition are somewhat less susceptible to viral and protozoal infections than those in a good state of nutrition--that is, to the rate of spread of a virus or protozoa within the body. This is not necessarily related to the immunological response of the animal but seems to be related more to the condition of the tissue cells themselves and to the state of dehydration of the animal.

Change of Diet as It Affects Susceptibility to Infection in Mice

Experimental use of mice has repeatedly shown that fasting for 18 to 24 hours increases the susceptibility of mice to infection with Salmonella enteritidis, K. pneumoniae, and Myco. tuberculosis. This period of fasting in a mouse, an animal with a high rate of metabolism, is a more severe procedure than the same length of time applied to a larger animal. Reduction of the food intake, however, also has been found to have the same effect. The increased susceptibility to reduced food, however, does not persist, and the resistance of mice tends to return to normal after a short period even if the inadequacy is allowed to continue. The mice may be said to have come to a new equilibrium. Until the animal becomes accustomed to its new food, a change in the ration is often followed by a few days of inadequate food intake, and this period is clearly one in which susceptibility to bacterial infections may be increased. The reverse situation presumably may also occur when mice are transferred to a foodstuff that is taken eagerly and results in overeating with subsequent disturbance due to excessive and unaccustomed food in the alimentary tract.

Whether overeating as opposed to inadequate nutrition alters the susceptibility of tissues as such to bacteria or to their toxins has not been explored, but as the equilibrium is upset--although in a different way--it is possible that this may be so, but the immediate effect is unknown. Time, however, is needed before any enhanced resistance conferred by a very good ration, with protein of a high biological value, is felt. A comparison of such a ration may show no difference between mice maintained on it and mice on the original diet a week after the change, although a difference can be found 14 or 21 days after the change of diet.

Many have sought to clarify the way in which dietary differences affect susceptibility to infection by studying the immunological responses. We have seen that no very conclusive result has been obtained as far as vitamin B deficiencies are concerned, and investigations concerning more general dietary conditions do not help to clarify the situation.

In a group of some 25 malnourished and wasted patients, the antitoxin response to injected diphtheria toxoid was far greater than in 18 healthy, well-fed patients (1). Measurements of blood volume indicate that the quantity of circulating antitoxin in the malnourished patients was far greater than in the well-nourished patients, even though the total of circulating plasma proteins was greater in the well nourished. In contrast to this investigation, there are many claims that protein depletion adversely affects antibody formation. Studies of Cannon (6) are possibly the most convincing.

The picture concerning acquired immunity is obscure, but one series of experiments illustrates that although the diet and the state of nutrition of the animal or of man affect resistance, the resistance conferred is different for different antigens, so that no generalizations are possible. The experiments are those of Melander and Oberg (17) and of Oberg and Melander (18). These investigations used a very delicate test of the effect of diet on antibody production in lambs and kid goats. The antigen used was a concentrated strain of influenza virus, which was given intraperitoneally to lambs or kid goats that were fed either on cow's milk or on homologous milk. Twins were used wherever possible. Lambs sucking the ewe made better weight gains than lambs fed by bottle on cow's milk. Consequently, for these animals there is some doubt whether the results were due to the better nutritional state or to the fact they were receiving ewe's milk instead of cow's milk. However, kid goats bottle fed with goat's or cow's milk, had similar weight gains, so that this doubt disappears. The titers for the plasma taken from lambs and kid goats receiving ewe's or goat's milk, respectively, were higher than those of lambs and kid goats receiving cow's milk, and the difference was consistent with time after the injection up to a maximum of 83 days. Three experiments were conducted with lambs and this difference was clear in two of the three experiments, whereas a clear difference was found in the more satisfactory experiment with kid goats.

A similar experiment with kid goats in which the antigen used was diphtheria toxoid produced no such difference, so that it was the nature of the antigen that determined whether or not a dietary effect was detectable. This experiment offers some explanation for the many contradictory reports in the literature.

Effect of Intestinal Bacteria or Endotoxins on the Susceptibility of Mice to Pathogens

Dubos and Schaedler (8, 9) showed that one normal inhabitant of the alimentary tract of mice may affect the susceptibility of the host to test infections. They observed that resistance conferred by dietary proteins or by proteins supplemented with amino acids to experimental infection with Klebsiella or Staphylococcus did not appear to be due to the rate at which the test organisms were eliminated from the tissues. This rate was estimated roughly by colony counts taken from homogenized organs of mice killed at different intervals after infection. The results revealed little difference between mice maintained on these rations. Instead, it seemed as if the tissues of the mice were not resistant to the organisms themselves but to their products (19).

To test this hypothesis, the endotoxin of Serratia marcescens (previously known as Bacillus prodigiosus) was prepared and small, graded doses were given to mice fed on diets containing 20 percent, 8 percent, and 5 percent casein, which were challenged by 0.05 ml. of a suspension of Staph. aureus given intravenously. The results showed (1) that at all levels of casein in the diet, the injection of endotoxin increased susceptibility to Staphylococcus; (2) that susceptibility increased as the endotoxin dose was increased; and (3) that susceptibility increased as the protein level was decreased. Tests with the endotoxins of K. pneumoniae and Escherichia coli showed that the susceptibility to these endotoxins as such was affected by the protein content of the diet when a mixed pelleted ration (24 percent protein) and a protein, low-corn ration were used.

The injection of endotoxin of Ser. marcescens similarly reduced resistance of mice to Staph. albus, which is normally avirulent to mice, and the effect was enhanced by low-protein diets (5 percent casein or corn diet). The lack of resistance conferred by wheat gluten was also confirmed in this experiment. The addition of endotoxin to such experimental infections did not appear to affect the spread of the infective bacteria in the tissues of the mice so far as could be detected by colony counts.

The work on the effects of endotoxins has given rise to a new line of thinking, which has revealed that the presence of subclinical infections within the alimentary tracts of mice may markedly affect their resistance to challenge doses of Staph. aureus and K. pneumoniae. Dubos and Schaedler (9) derived a "disease-free" strain of mice, which were maintained in strict isolation, by caesarian section from stock Swiss Albino mice. The "disease-free" mice, which must not be confused with "germ-free" mice, proved to have much greater growth rates than the stock Swiss Albino mice, although qualitatively the same differences in resistance appeared when they were tested while consuming the casein, beta wheat gluten, protein and low-corn rations, or a stock pelleted ration. These mice, however, proved to be much more susceptible to challenge doses of K. pneumoniae, Staph. aureus, Myco. fortuitum, and Myco. tuberculosis than the stock Swiss Albino strain, and this was true irrespective of the diet or of the route of infection. When the endotoxins of K. pneumoniae or of E. coli were tested, however, the "disease-free" mice were apparently entirely resistant to these endotoxins and survived six times the LD_{50} dose of the parent strain of Swiss Albino mice. On the other hand, relatively minute doses of these endotoxins increased the susceptibility of the "disease-free" mice to Staph. aureus given by intraperitoneal injection, and it was concluded that the endotoxin contained an unidentified material that enhanced susceptibility to Staphylococcus, but which had no other effect on the mice.

Examination of the intestinal contents of the two strains of mice revealed that they were different in that a lactose-fermenting coliform organism, tentatively identified as E. coli, was consistently present in the Swiss Albino mice but absent from the "disease-free" mice, irrespective of the diet.

The "disease-free" mice rapidly acquired E. coli in their intestines when placed in contact with infected Swiss Albino mice. Subsequent tests with these animals showed that their susceptbility to Staphylococcus or to K. pneumoniae and to the endotoxins of K. pneumoniae or to E. coli was now no different than that of the Swiss Albino parent strain of mice.

These experiments provide much food for thought and indeed make it necessary to reappraise many experiments of the past. Infection with this E. coli organism alone was sufficient to alter the otherwise "disease-free" mice so that they were indistinguishable from the Swiss Albino strain. However, other changes were also noted in the intestinal flora, and it would be unwise to assume at present that E. coli was the only organism concerned. The fact that minute doses of endotoxin in "disease-free" mice rendered them as susceptible to Staphylococcus as the Swiss strain is perhaps an indication that the acquisition of an intestinal organism such as E. coli in any animal not previously exposed could trigger off a latent infection of Staphylococcus.

There is no need for me to point out the importance of Staphylococcus in the health of farm livestock or the mystery that surrounds the occurrence of clinical staphylococcic conditions. However, this takes us far away from nutrition unless it can be proved that incidence of subpathogenic forms of bacteria in the intestines of domestic animals is affected by the food eaten by the animal.

Effect of Nutrition on Certain Conditions of Domestic Livestock

There seem to be comparatively few accounts of nutritional studies as such on the susceptibility of poultry or livestock to bacterial and virus infections. As far as parasitic infections are concerned, there seems to be little doubt that animals in a poor state of nutrition are less able to combat the effects of parasitic invasions with nematodes than animals in a good state of nutrition.

Work has been done on the effect of diet on the susceptibility of chickens to infection with Salmonella gallinarum. Williams-Smith (30) compared the ability of chicks raised on whole wheat, ground wheat, and a mash containing 10 percent fishmeal to withstand avian typhoid produced by Salmonella. Whole or ground wheat diets allowed greater survival than the mash containing fishmeal in birds exposed to infection. Examination of the contents of the crops, gizzards, and intestines of birds on these rations showed that the acidity of the gizzard contents was significantly higher in birds fed on mash containing fishmeal than in those fed ground or whole wheat--pH 3.7 to 3.8 as opposed to 2.5 to 2.7.

Tests in which Salmonella was added to the food acidified to different pH values showed that at pH 2.5 most of the organisms were killed within 20 minutes or less, whereas at pH 3.5 large numbers survived for 40 minutes or longer. The contents of the duodenum, jejunum, and ileum of the chicks showed that rapid proliferation took place when surviving bacteria reached the intestines. Williams-Smith proposed that as whole wheat took longer to traverse the proventriculus and gizzard, and as the acidity of the contents was such that practically all organisms would be killed in a short time, the resistance conferred on chickens by whole wheat as opposed to mash containing fishmeal was due to the greater acidity within the proventriculus and gizzard, with possibly a longer exposure of food and, consequently, organisms to this environment. He examined the composition of the mash mixture to see which part of it neutralized acidity and found that fishmeal was responsible. Casein had no such effect. He also showed that adding alkaline materials such as calcium carbonate to the wheat diet or to ground wheat decreased resistance to Salmonella.

This is an interesting and simple example of diet affecting resistance to infection but is resistance quite the right word here? Resistance implies that the alimentary mucosa or the tissues themselves are less susceptible to bacteria or to their products rather than that the bacteria are killed in such a simple way by gastric juice.

I quote this experiment because Salmonella infections have been so extensively used in mice to test dietary effects, and most workers have been content to give their test doses orally. Only Webster (25) in examining genetically resistant or genetically susceptible selected strains of mice has tested the subsequent dietary effects by both oral and parental infections with Salmonella. In his genetically selected strains the responses to both forms of administration were similar, indicating that in these mice the animal tissues themselves were either more or less resistant to the infective organisms.

It should also not be overlooked that diets containing a high proportion of wheat have proved superior to synthetic rations in mice as regards conferring resistance to infection, and this has led to a hunt for a resistance-conferring factor, which was found to reside in the wheat germ (20). No further information on this factor seems to have been published.

It may be that the simple gastric effect found by Williams-Smith (30) in chickens is only partly concerned with any favorable effect conferred by the wheat diet, for he failed to test resistance to Salmonella by injecting the organism. However, it may also be that the elusive wheat-germ factor postulated by Schneider (20) could be partly explained in a simple way on the lines suggested by Williams-Smith (30).

Hill and Garrow (12) also studied the protein content of the ration in relation to chicken resistance to Salmonella gallinarum. They found that casein or soybean meal gives less resistance as the quantity in the diet is increased from 10 to 30 percent. They did not test any alimentary effect and this may be a similar phenomenon to that observed previously by Williams-Smith (30). However, he found that 15 percent casein in the ration was equivalent to whole wheat in conferring resistance. The experiments are not really comparable.

When dealing with alimentary infections of birds or animals having a complicated gastric system, it is important to search for alimentary effects as well as to test whether the alimentary mucosa or the animal's tissues themselves are rendered more or less resistant according to the food fed. In the ruminant, infective organisms ingested by mouth will first have to survive the competition of the dense population of nonpathogens in the rumen. The survivors then have to traverse the abomasum and the acid environment within it before passing to the intestines where conditions are favorable for growth. It is enough to say that the acid environment of the abomasum--pH 3.0 or thereabouts--is sufficient to reduce a viable population of starch-fermenting organisms from 10^7 to 10^4 or 10^5 A similar death rate may be expected in many pathogens.

Bullen (3), on examination of sheep slaughtered in the local abattoir, found that in 21 percent, the rumen contents contained Clostridium welchii type D, whereas this organism was present in the whole alimentary contents of 41 percent of these animals. Of the cultures isolated, 55 percent produced epsilon toxin.

Later when studying the experimental induction of enterotoxemia, Bullen and others (5), noted that when cultures of C. welchii type D were introduced into the rumen of healthy sheep, there was apparently a heavy death rate as the concentrations fell a great deal more rapidly than the concentration of a marker introduced at the same time. The concentration of C. welchii entering the duodenum was also far lower than that in the rumen.

The work of Bennetts (2) and of Harshfield and others (11) has shown that it is the best animals that succumb to enterotoxemia. The same is true of lamb dysentery. Bullen and Scarisbrick (4) went to considerable trouble to understand the conditions that allowed sufficient growth of clostridia organisms to occur in the small intestine to produce lethal quantities of toxin. They found that sudden excessive wheat feeding would cause enterotoxemia, and in animals that succumbed to enterotoxemia large quantities of starch, which were absent normally, were found in the intestine. The presence of starch was associated with large numbers of clostridia organisms. Under these circumstances toxin production was abundant and death ensued.

The production of enterotoxemia, therefore, demands first the presence of the organism in the gut and, second, sufficient substrate for its rapid growth. Diet clearly plays an important part in the second factor. The ingestion of excessive quantities of starch or glucose or wheat, as shown by Hungate and others (15) and by Turner and Hodgetts (22), produces an acidemia usually associated with an excessive lactic acid fermentation in the rumen. That such an acidosis can be fatal in itself--quite apart from any complication by clostridia--confuses the picture. Bullen and Scarisbrick (4), however, have shown that when acidosis is prevented by feeding alkali or by suppressing fermentation in the rumen with penicillin, enterotoxemia can still occur in the presence of large quantities of starch in the intestine, provided clostridia are present.

Harshfield and others (11), however, found that enterotoxemia was usually associated with a sudden change of diet, especially when lambs were put in lucerne pastures. Bullen and Scarisbrick (4) suggest that under these conditions, the rumen may be swamped with unaccustomed quantities of fermentable carbohydrate, enough of which pass intact to the intestines and allows the proliferation of any clostridia also present. In other words, it takes some time for the rumen flora to adjust itself to sudden changes of this nature, and in the process less food is fermented than would occur once adjustment has taken place. This is really saying that the equilibrium established between the host, its alimentary flora, and the pathogens is disturbed by sudden change of diet, and that the pathogens are then in a more favorable position to proliferate in the intestine, and consequently to produce toxin.

Apart from fowl typhoid and enterotoxemia there seems to be little direct evidence from farm livestock of the effect diet may have on infectious disease. A report by Luecke (16) suggests that in pigs with certain vitamin B deficiencies--he reports on pantothenic acid deficiency--resistance to Salmonella choleraesuis is reduced.

In conclusion it can be said that the subject of nutrition in relation to infections is still bedevilled by obscurities. There are clear-cut experiments that show dietary effects, and I have quoted those that seem to be most enlightening. Good nutrition is usually taken to mean the dietary conditions that allow greatest growth and weight gains and the greatest reproduction of the species concerned. It is quite obvious, however, that these criteria are inadequate to adopt as indicators of resistance to infection.

Howie and Porter (14), using diets composed of natural foodstuffs, observed in mice that the diet that was best in regard to reproductive efficiency was among the worst in conferring resistance to tubercular infection and Salmonella. Others have made similar observations. Dubos and Schaedler (9) showed that mice in a disease-free state are highly susceptible to staphylococcic infection, although their nutritional performance was exceptionally good. Experiments such as these should be a warning against generalization.

Diet is a factor that cannot be ignored, and it may have a marginal effect on the outcome of any exposure to infection. As was shown by Schneider and Webster (21), dietary effects are completely lost in mice exposed to a virulent bacterial infection. In animals with a complicated alimentary system, such as the ruminant and the chicken, simple alimentary effects are likely to be important as regards alimentary infections. However, the demonstration of Dubos and Schaedler (9) of the effects of one common alimentary organism on the resistance of its host to experimental infection opens a new and fascinating approach to the subject.

Further work by Dubos and Schaedler (10) revealed that the concentrations of lactobacilli in the fecal pellets of mice fed on synthetic diets are small when compared to those from mice fed on a diet consisting of unpurified foodstuffs. The resistance to experimental infections of Staph. aureus (strain Giorgio) and Klebsiella pneumoniae type C was greater in mice fed diets containing unpurified foodstuffs than in those fed the synthetic rations. The authors pointed out that their experiments do not provide any evidence that the presence of an abundant flora of lactobacilli in the gut and the animal's resistance to infective organisms are causally related. Further work is necessary to decide whether the association is fortuitous or not.

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THE ROLE OF CHEMICALS AND HORMONES IN INFLUENCING HOST SUSCEPTIBILITY

Alvin S. Levine, Ph. D.[1/]

The state of good health and a condition known as disease are sometimes difficult to separate. Many factors contribute to well being. The body's chemicals are constantly being turned over in metabolic processes that are best understood when explained biochemically. Even mental health is gradually giving way to biochemical expressions.

Just as disease is associated with micro-organisms, so is the micro-organism's ability to disturb the host attributed to a variety of virulence factors. These factors either resist the host's defense mechanisms or harmfully affect the host.

Infection can be regarded as a special case of parasitism. The host organism may serve as substrate for the pathogenic micro-organisms's enzymatic systems. Defense measures of the host may also include enzymes. In the event of infection, it is important whether or not these enzymes find a substrate in the invading micro-organisms. From this point of view, a disease occurs when the balance between parasite and host becomes disturbed. The parasites multiply, and in injuring the host they produce the picture of an infectious disease. If an equilibrium is established, we speak about subclinical infection or perhaps latent infection. The presence of infection is the usual case, and the general health of the host is the significant point in this discussion.

Adaption to environmental alterations and the ability of self-regulation are fundamental to all living things. Claude Bernard first conceived the concept of the constancy of the internal environment, now known as the dynamic steady state or homeostasis, as resting on the maintenance of an equilibrium of systems of the organism (1, 2).[3/]

1/ School of Medicine, University of Indiana, Indianapolis, Ind.

2/ The oral presentation was accompanied by 11 additional tables that are not included in this publication.

3/ Numbers in parentheses refer to Literature Cited at the end of this paper.

The ability for self-regulation is the capacity to alter the rate of metabolic reactions. Since most of the metabolic processes in the organism are mediated through the activity of specific enzymes, it is necessary to consider the role of enzymes in resistance and susceptibility to infection.

To maintain a homeostatic dynamic steady state, there exists a continual demand for energy, which is derived directly or indirectly from the metabolism of foodstuffs. Metabolic homeostasis depends on the controlled release of energy, and alterations in this process in the host or micro-organism result in its inability to adapt to a changing environment. Of the many factors involved in regulated metabolic reactions, minerals and vitamins act as coenzymes in enzymatic reactions.

Hormones affect the rates of metabolic reactions by regulating the synthesis of enzymes. This was first demonstrated by Sutherland and Cori who showed that epinephrine and the hyperglycemic factor of the pancreas accelerate the synthesis of active phosphorylase in liver. The role of foodstuff and hormones on enzyme levels has been demonstrated by the effect of fasting on liver carbohydrate enzymes in normal rats and in hypophysectomized rats. Loss of enzyme activity results from fasting, but, in the absence of resynthesis in hypophysectomized animals, more rapid loss of enzyme activity is demonstrated.

In different infections, different enzymes take an important part; the function of most of them is not yet clear. Among these disputed but certainly important enzymes, one might include hyaluronidase, which plays a part in streptococcal and staphylococcal infection. Lecithenase, the alpha toxin of Clostridium welchi and Clostridium perfringens, was the first enzyme shown to be an active toxin. The effectiveness of phagocytosis as a mechanism of host defense is primarily attributed to its battery of enzymes capable of digesting the micro-organism. Those micro-organisms capable of using the phagocyte as its substrate usually are endowed with capsules or cell-wall material resistant to the enzymes of the phagocytic cell. The concentration of serum lipase was at one time thought to explain the species selective resistance of the rat and rabbit over the guinea pig to infection by the tubercule bacillus. However, on examination it turns out that the rat has the lowest serum lipase level and the guinea pig the highest (3).

Variation in normal levels of enzymatic activity remains an attractive hypothesis for susceptibility to specific infectious agents. The relative activities of various liver enzymes in the chicken, rat, and man give some indication of variation among the species. These enzymes are compared in table 1.

TABLE 1.--Comparison of activities of liver carbohydrate enzymes in the chicken, rat, and man[1/]

Enzyme	Enzymatic activity[2/]		
	Chicken	Rat	Man
	Percent	Percent	Percent
Phosphohexoseisomerase	100	100	100
Lactic dehydrogenase	58	139	28
Phosphoglucomutase	2.3	65	65
Glucose-6-phosphatase	-	4.3	1.6
Fructose-1, 6-diphosphatase	1.7	2.8	.9
G-6-P dehydrogenase	.03	.6	.6

1/ For additional information see the work of G. Weber (18).

2/ Enzymatic activity was calculated as a percentage of phosphohexoseisomerase, which was arbitrarily taken as 100 percent.

An example of how an inorganic chemical could have a role is in cell injury, which results in liberation of potassium. It is reported that K^+ convert resting wandering cells into active macrophages in mobilizing cellular defense mechanisms (4, 5, 6). On the other hand, the role of inorganic ions on influenza virus infection in vitro is well documented for increasing susceptibility. Whereas Ca^{++} is required for adsorption of virus to the host cell, the presence of K^+ is necessary for penetration of the cell (7). It is proposed that influenza virus does not actively penetrate the host cell, but that the cell membrane actively takes in the virus. In the absence of K^+, the adsorbed virus remains on the surface to become denatured or neutralized. Once it is inside the host cell, it is protected from such effects as neutralizing antibody and can go about its business of diverting host cell enzymes to making more influenza virus.

Potassium is required in a number of metabolic reactions. It is directly concerned in the action of the enzyme pyruvic phospherase, which catalyzes the reaction:

phosphoenol pyruvate + adenosine diphosphate
pyruvate + adenosine triphosphate

Antagonism of Ca^{++} and Na^+ to K^+ in the above reaction is reported.

Phagocytosis appears to be a generalized phenomenon. Organic or inorganic particles are taken in without unusual selectivity. The ability of microorganism to multiply in a cell may depend on the metabolic pattern present. A change in the carbon source of energy for chorioallantoic membrane in vitro will demonstrate the specificities that are required with two different strains of influenza virus (8). The data to be presented suggest that there is a metabolic basis to tissue tropism. It is proposed that a virus or other type of microorganism multiplies in a particular tissue, because the biochemistry of various tissues are different, and some are better suited for the agent than others.

Experiments (8) have used modified Hanks' balanced salt solution (BSS) in all systems as the basal medium for maintenance of minced chorioallantoic membrane from 10- to 12-day-old embryonating chicken eggs.

Modified Hanks' Balanced Salt Solution

NaCl	8.0 mg./ml.
KCl	.4
$MgSO_4$ ($7H_2O$)	.2
$CaCl_2$	.14
Na_2HPO_4($12H_2O$)	.06
KH_2PO_4	.06
$NaHCO_3$	1.0
(Glucose and/or Na Pyruvate concentration modified as specified)	
Phenol Red	.02 percent
Penicillin	10 units/ml.
Streptomycin	10 units/ml.

The effect of pyruvate and/or glucose on multiplication of strain WS and the neurotropic strain NWS of influenza virus was observed. The addition of 0.1 percent pyruvate without glucose resulted in multiplication of strain WS of influenza virus to give a hemagglutinin titer of 1/16 within 48 hours. In the case of strain NWS, hemagglutinins were not detected when pyruvate was the sole carbon source in the medium. The substitution of glucose as the sole carbon source permits multiplication of both viruses. Although pyruvate was not a suitable carbon source for multiplication of the neurotropic virus, when added with 0.02 percent glucose, which by itself does not permit multiplication of strain NWS, a synergistic effect is observed. It would seem that glucose contributes something to these cells that permits a selective effect on multiplication of strain NWS of influenza virus.

Infectivity titers are compared in table 2. Although hemagglutinins are a useful assay of viral multiplication, the infectivity titration is the true measure of virus. Results following various time intervals after inoculation of a thousand infectious units of virus are given for both strains WS and NWS when 0.1 percent pyruvate was the sole carbon source in the balanced salt medium.

Tissues infected with strain WS and homogenized at 12 hours show egg infectivity titers of $10^{4.4}$ and $10^{6.4}$ after 48 hours. Tissue data for strain NWS show some evidence of intracellular multiplication, which may result from the endogenous metabolism of the cells rather than from pyruvate. However, insufficient virus is synthesized for detecting NWS virus in the supernatant fluid by hemagglutination. Efforts to select a line of virus from the chorioallantoic membrane tissue culture system that would multiply in tissue maintained in pyruvate was without success, as blind passages of supernatant fluid at 24-hour intervals failed to yield virus.

If other strains of influenza virus are compared in the selective nutritional medium, strain PR-8 of influenza virus multiplies equally well with glucose or pyruvate as the sole carbon source. Other strains of influenza virus do not give equal results. Nuerotropic strains, as previously demonstrated, multiply better with glucose as the sole carbon source as indicated by an index greater than one. Influenza B has some preferential ability to grow in glucose over pyruvate as a carbon source. Swine influenza virus apparently gives some indication of an apposite effect, as higher hemagglutinin titers are observed with pyruvate than with glucose. Since Newcastle disease virus is also a myxovirus having a mouse brain-adapted line (Iowa 125 mouse passage), neurotropism per se was not found to be the basis of the pyruvate effect on strain NWS of influenza virus. At this time, it is not known why strains WS and NWS show differnces in respect to multiplication in mouse-brain or in tissue-culture systems of different carbon sources.

Not only the carbon source or the nutritional aspects influence viral multiplication, but the age of the chorioallantoic membrane also has a selective effect. When chorioallantoic membranes are from eggs that have been embryonated 8 to 12 days as compared with membranes that are taken from eggs incubated 15 days at 39° C, strain NWS of influenza virus does not multiply in 15-day-old membranes whether glucose or pyruvate is the carbon source. It is important that strain WS multiplies in the presence of either glucose or pyruvate, since evidence shows the physiological good health of the cells and the presence of all essential factors for multiplication of influenza virus. Carbohydrate metabolizing enzyme activities do not show great differences when 10- and 16-day-old chorioallantoic membranes are compared on a cellularity and nitrogen basis. The activities of the following enzymes were determined: Phosphohexoseisomerase, lactic dehydrogenase, phosphoglucomutase, 6-phosphogluconate dehydrogenase, fructose-1, 6-diphosphatase, and glucose-6-phosphatase. It is quite possible that other enzymes' systems, which have not been tested show marked differences. Along about the 14th or 15th day of age, chorioallantoic membrane is reported to have a reduced steroid level. This may be significant, but in vitro tests of several steroids on multiplication of influenza virus in chorioallantoic membrane have not shown differences. The studies are being continued.

TABLE 2.--Effect of 0.1 percent pyruvate medium on multiplication of virus strains WS and NWS in cultures of minced chorioallantoic membrane

Hours after inoculation	WS strain			NWS strain		
	Tissue	Supernatant fluid		Tissue	Supernatant fluid	
	EID_{50}	EID_{50}	HA 1/	EID_{50}	EID_{50}	HA 1/
0		10^{2}			10	
1		10^{2}			< 10	
6	$10^{1.7}$	< 10		$10^{2.3}$	<10	
12	$10^{4.4}$	$10^{2.3}$		$10^{2.3}$	<10	
24	$10^{4.5}$	$10^{5.5}$	<2	$10^{4.2}$	$10^{1.8}$	≤2
48	$10^{6.4}$	$10^{4.4}$	4	$10^{3.6}$	$10^{1.6}$	≤2
72			8			<2

1/ Hemagglutinin titer was the reciprocal of three experiments, pooled.

The glucose metabolized in infected and uninfected 12-day-old chorioallantoic membranes was studied. The data showed that infection at time zero with 16 hemagglutinin units of virus resulted in multiplication of both strains WS and NWS of influenza virus as indicated by an increase in final hemagglutinin titers. The milligrams of glucose metabolized per 100 milligrams of chorioallantoic were recorded. There was little difference between the glucose metabolized by strain WS infected and uninfected tissue. However, strain NWS-infected tissue showed a marked increase in glucose metabolization. Total oxidized glucose CO_2 did not show a difference in uninfected tissue or infection with either strain of virus.

Pyruvate metabolism was observed in tissue cultures infected with NWS and WS strains. The infected cultures showed a marked increase of pyruvate metabolism over the uninfected or control cultures. However, strain NWS does not multiply in chorioallantoic membrane maintained in pyruvate as the sole carbon source.

Strain WS of influenza virus-infected chorioallantoic membrane maintained in C^{14}-glucose showed a marked increase in lipid C^{14}, whereas protein C^{14} did not show a marked change. It would seem more reasonable if tissue infected with strain NWS had shown increased lipid synthesis, since glucose utilization data suggested synthesis. The composition of influenza virus is ribonucleic acid, lipids, and carbohydrates. Models of its reproduction in chorioallantoic membrane suggest assembly-line activities of nucleus, cytoplasm, and cell membrane in production of complete or infective virus (9). Influenza absorbs onto the surface of chorioallantoic membrane cells in the presence of calcium ions. Penetration takes place in the presence of potassium ions. There is a dark phase during which the virus cannot be demonstrated and the nucleic acid finds its way to the nucleus of the cell. After about 3 hours, an increase in soluble antigen can be demonstrated by fluorescent antibody staining in the nucleus of the cell. At about 4 hours hemagglutinins are evident in the cytoplasm in the absence of infective virus. Electron microscopic evidence of virus budding from the cell membrane can be demonstrated at about 6 hours, and infective virus is present. The complexity of synthesis of influenza virus and the interrelationship of activities in the nucleus, cytoplasm, and cell membrane would suggest that the balance of the separate functions of the machinery of the cell is critical. A selective metabolic antagonist inhibits virus synthesis through inhibition and alterations of metabolic patterns of the cell. It is not unreasonable that cell susceptibility and resistance to infection is a finely balanced state which reflects the response of the host to infectious agents.

When one reviews the literature of the role of hormones on susceptibility to infection, the most contradictory of results are found. In a recent review (10), it is pointed out that diabetes is generally thought to increase susceptibility to infection. However, little evidence supports this view. Kass (10) suggests that the concern over infection in cases of diabetes mellitus, when placed in proper perspective, is based on the influence of infection on control of diabetes. Severe infections may complicate diabetes by disturbing carbohydrate, mineral, and other aspects of metabolism which affect the well-being of the host. Although there are reports of more extensive surface infections such as caused by fungi, the cause of death in diabetics does not reflect infections as being responsible. Further, the formation of antibody in alloxan diabetic rabbits was not altered (11).

Corticosteroids have increased the rate of synthesis of a number of enzymes. However, this increase has depressed antibody formation with excellent clinical use in the control of hypersensitivities and collagen diseases. In a survey of 100 patients with severe sepsis, Kass and Finland (12) found the blood level of corticosteroids above the average. This could be responsible for selectively suppressed antibody formation, or as proposed by Kass, it could be caused by an impairment of ability to metabolize steroids. Studies with adrenalectomized mice and pneumococcal infections show selective effective dose range for survival of mice. Too little cortisone was ineffective and too much cortisone was also ineffective. Another important consideration is the dissociation of toxic action of bacterial or host reaction products from other aspects of resistance to infection. In contrast to the selective dose range of cortisone for protection of mice from pneumococcal infection, animals receiving challenge inoculation with endotoxin derived increased protection as the dose of the corticosteroid increased. There are also apparent differences in the anti-inflammatory and anti-endotoxic effects of corticosteroids.

Studies of the influence of ovarian hormones on uterine infection in cattle by Rowson and others (13) showed that the bovine uterus during estrus appears remarkably resistant to infection, but during the luteal phase, it is most susceptible to infection, and pyometritis frequently results. In experiments on ovariectomized cows, progesterone was shown to promote conditions suitable to the growth of bacteria, whereas exogenous estrogen renders the bovine uterus resistant to infection. These data were considered the basis for a cow's recovery spontaneously from infection after several heat periods without reinfective coitus. They also show why the bull does not recover spontaneously, since testosterone has a systemic effect similar to that of progesterone in the cow. Pyelonephritis as a complication of pregnancy in the human was found by Kass to have a similar pattern by control of the bacteriuria with antibiotics during pregnancy. The associated high incidence of prematurity and neonatal death was also reduced.

In a general way, hypothyrodism increases susceptibility to infection, and hyperthyroidism acts inconsistently in the opposite direction. There is reported no major thyroidal influence on antibody production, phagocytic mechanism, or the inflammatory process.

Of the other hormones, the little data available offer no clearcut patterns.

In this discussion, I have tried to build a case for metabolic patterns as the basis for susceptibility to infection. If hormones regulate metabolic patterns by controlling enzyme synthesis, virus should be useful as test organisms in that they are completely dependent on the host enzyme systems.

Other experiments showed the influence of cortisone on the course of influenza infection in mice.

The effect of cortisone on pneumonia of mice infected intranasally with mouse lung-adapted PR-8 strain of influenza virus was studied. The method of calculating the percentage of pneumonia was the lesion score assay of Horsfall. Cortisone-treated mice showed an increase in percentage of pneumonia when compared with saline-treated control mice (14). These results were observed over a wide range of infecting doses on the fourth day following infection.

The effect of cortisone on infectivity titers of strain PR-8 influenza virus in mouse lung was observed. The appearance of infectious virus in lung tissue was observed earlier and virus titers remained higher through the fourth day in cortisone-treated mice than in saline-treated mice.

The effect of cortisone on dissemination of influenza virus strain PR-8 to extrapulmonary tissues in mice was studied. It was observed that virus is isolated from liver of cortisone-treated mice on the fourth day. Cortisone also induces dissemination to spleen on the sixth day following infection. The effect of cortisone on experimental influenza in mice showed increased viral synthesis as indicated by the earlier appearance of virus in lung tissue and by the more rapid onset of pneumonia. Earlier dissemination of virus to liver and the appearnace of PR-8 strain of influenza virus in spleen was additional evidence of the influence of cortisone on the course of infection. However, different strains of influenza virus showed dissemination patterns, which are characteristic of the virus strain (15).

A comparison was made of infectivity titers of several strains of type A influenza virus in lung, liver, spleen, kidney, and brain tissues after intranasal inoculation of virus. It was necessary to sacrifice mice infected with 5,000 infective doses of strain WS after 2 days because of its virulence. These mice would die within 2 to 3 days. Comparison with other strains, 2 days following intranasal infection showed only strain WS to disseminate to the kidneys as well as to the liver. It is of interest that mouse brain-adapted neurotropic strain FNWS does not disseminate to liver, whereas mouse lung-adapted neurotropic strain FNWS disseminates to the liver. A hundredfold reduction in infective dose permits strain WS-infected mice to survive 4 days. Comparisons at this time show the dissemination of mouse brain-adapted strain FNWS to spleen tissue and the absence of dissemination of mouse lung-adapted strain FNWS or strain WS to the liver.

The relationship of patterns of dissemination to virulence has not been studied. However, cortisone alters the course of PR-8 infection in mice to give a different disease pattern. The mechanism of the cortisone effect is not known, but cortisone has been shown to give increased transaminase activity in liver suggesting that more keto-acids are formed and increased gluconeogenesis occurs (16). Increased glucose synthesis from pyruvate in rat liver has been observed in cortisone-treated rats (17).

The data presented in these studies suggest that there is a metabolic basis to the selective nutritional effects on viral multiplication as well as tissue tropism or dissemination patterns during infection or both. Perhaps the role of chemicals and hormones in susceptibility can be better understood in the light of these considerations.

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THE IMPORTANCE OF SCIENTIFIC RESEARCH FOR IMPROVING LIVESTOCK PRODUCTION AND HEALTH IN HIGH ALTITUDES

Teodoro Ramos-Saco, V. M. D. 1/

Throughout the world, there are extensive mountain systems such as the Himalayas, Rockies, Andes, Alps, and others. The higher altitudes vary in distance above sea level, as do the lower altitudes where man exists and nature permits the survival and rearing of livestock. Great areas of the earth are not used for agriculture. In some areas, however, the conditions in higher altitudes are a challenge to livestock production. Such conditions are particularly important to the American continents as there are extensive mountainous regions.

Besides the difficulties encountered in using these areas profitably for such industries as mining and in attracting the large investments needed for transportation facilities, the nature of the environment creates its own special obstacles to agriculture and livestock. However, the rapidly growing population makes it mandatory to improve land utilization. The potential for livestock and agriculture has not been properly used. This lack of progress is the prime cause of stress in underdeveloped countries and is responsible for serious economic and social effects. Proper use of these high-altitude areas can only be carried out through basic research of the fundamental problems. Although man is desperately seeking more knowledge of outer space, he has acquired little knowledge about the high altitudes of the very earth on which he depends for health and food.

Physical Environment

Many varieties of physical environment are created because of the great range in altitude over the earth. These environments must be considered, not only from the viewpoint of barometric pressure but also from daily temperature range, rainfall, humidity, oxygen tension, light, radiation, and other factors. Extreme differences in the normal calculated figures for these factors are observed as we approach the peaks of the highest mountains.

The snow-capped mountain peaks are a constant source of water for the rivers and, along with the rainfall, form the basis for agriculture. Rainfall is not evenly distributed throughout the year. Seasons of heavy rainfall are often followed by long seasons of drought that endanger livestock.

1/ College of Veterinary Medicine, University of San Marcos, Lima, Peru.

Daily temperatures range widely in the high altitudes. For example, livestock in the Andean region of South America endure daily temperatures that vary some 72° F. between the high and low for the day.

Sheep and cattle rearing is common in altitudes of 13,000 feet above sea level where the atmospheric pressure is 430 mm., and oxygen tension, 86 mm. Llama and alpaca are found at this altitude and up to as much as 15,000 feet or more.

Light intensity at high altitudes is quite high during certain times, and the figures for ultraviolet and cosmic radiation are considerably higher than at sea level.

Agriculture and Livestock

The use of land for agriculture in high altitudes is limited by climate and soil fertility. The higher the altitude, the more severe the limitations. Legumes (alfalfa and clover) are successfully grown at altitudes of 10,000 feet, and even dairy farming is profitable under these conditions. At higher altitudes, legume growing is no longer productive, and only cereals may be grown. Rye is the hardiest cereal in high altitudes and poor soil. Generally cereal stalks and natural pastures are practically the only basic forage sources for livestock at elevations of 12,000 to 13,000 feet in the Andes. Thus, livestock rearing must be handled carefully because of the severe environmental conditions.

Most of the livestock raised in the high Andes are sheep and cattle that originated from animals brought in by the Spanish conquerors many centuries ago. There are also the native species--llama, alpace, and vicuna--found mainly in Peru and Bolivia and used for meat, work, and as a source of fine clothing.

As a result of their long history in the high altitudes, the native cattle have changed in morphology and physiology to adapt to the environment. Although their growth rate and production are quite poor, they have remarkable qualities of resistance to adverse conditions. Considering these qualities, one can see that there is an enormous potential at hand for genetic improvement and production, provided that adequate measures are taken for improving nutrition, sanitation, and management. Experience shows that, when we try to raise animal health and production, we must not introduce nonadaptable strains of stock, which might not survive or might produce susceptible strains. Artificial insemination for accelerating this process could be disastrous.

The native animal species--llama, alpace, and vicuna--reared at altitudes of more than 15,000 feet are the best example of adaptation to environment. The animal population in South America of these species reaches approximately seven million head.

It has been quoted (3)[2/] that "The organism is whole within an environment. It cannot be considered apart from that environment; knowledge of the environment is therefore as important as knowledge of the organism, and knowledge of the whole organism living in its environment is more important than the most intimate familiarity with all its parts."

This fundamental idea has been difficult to incorporate into the sciences and into sociology. The individual--whether plant, animal, or man--is the result of heredity plus environment.

In America, large populations have lived since prehistoric times at great altitudes above sea level. Have the special conditions of living and working at 6,000 to 15,000 feet left an identifiable imprint? This question, among others, clearly establishes the need for a study of Andean biology as related to man, plants, and animals.

In considering living organisms and their environment, we should not overlook the important role of soil as a source of nourishing life. Variations in the composition of soil may play an important part in determining the kinds of animals and plants that can exist in a particular place. The interaction of such factors in adaption is often highly complex, and many of these factors remain unidentified.

Climate has a great influence on soil quality. Soil fertility depends on physical, chemical and biological factors which are affected by climate. Besides temperature and rainfall, which are common factors influencing soil everywhere, attention should be drawn to the fact that low-oxygen tension in the air can also affect soil quality. Such is the case in high altitude regions, where oxygen tension in the atmosphere is about half that at sea level. As far as soil air is concerned, very little is known either about its composition or about the extent to which this fluctuates. The percentage of oxygen in the soil is always slightly less than that in the atmosphere.

It is known that the mobilization of nutritive chemicals for plant nutrition is affected by the influence of oxygen on chemical soil reactions. It is also known that soil micro-organisms require the use of oxygen for their life and propagation, and that other forms of higher animal life in soil require oxygen and, in their turn, add to soil aeration and fertility. But, to what extent do these activities take place under low-oxygen tension in soil air? What influence does atmospheric pressure have on soil air and atmospheric air? What are the consequences with regard to soil biology and plant growing? Here is a field of research which would undoubtedly repay further investigation.

2/ Numbers in parentheses refer to Literature Cited at the end of this paper.

Besides the effects that high altitude environment might have on soil fertility and its ultimate effects on plant growing and agriculture, it would be most interesting to know what are the direct effects on soil air. Unfortunately, this subject has as yet barely been touched.

Plant respiration, which is an essential feature of plant physiology, is based on oxygen and carbon dioxide utilization. But, to what extent does low-oxygen tension in the atmosphere impair plant respiration and plant physiology? What mechanisms of adaptation have taken place in order to counteract this factor? What morphological and physiological features have developed in order to perform the proper functioning of respiration under these conditions? Would native botanical species, long adapted to this environment, show marked differences in these features compared with other lowland species? Could these features be utilized as a measure of adaptation to low-oxygen tension and to high altitude? Should they be used genetically for plant selection for these conditions?

Native highland flora is rich in species that have to withstand the most adverse climatic conditions. Plants have to withstand a wide range of daily temperatures, which reaches very low points during winter nights, and to overcome long drought seasons. Only native flora is able to survive these conditions.

As this flora is the basic forage of livestock reared at high altitudes, it is most important to determine its nutritive value and yield. A great deal of experimentation has to be made in order to improve forage production under these conditions. Agronomic science and genetics must be applied in order to reach successful results.

As seasonal droughts are quite serious in some years, vitamin deficiencies are not unusual, and the seasonal plant variations deserve special study. Forage conservation and management are also subjects needing considerable experimentation in the interests of profitable livestock rearing. Copper and iodine deficiency has also been observed in some highland regions, but there remains the possibility that other minerals may also be deficient.

Research into these altitude problems, which affect animal health, warrant particular consideration. There is a great deal of work to be done, and the results would probably have as great an importance in this field as those of medicinal research. Veterinary research has moved at a very slow pace. Research into human physiology at these altitudes has received far greater attention. The results of human research could be quite enlightening when applied to the problems of animal physiology, and reference to this subject will be made later.

Altitude combined with known pathogenic elements creates a distinctive bioclimatology and, hence, new aspects of pathology. Among pathogenic microorganisms of animals, observations have been made in Peru on the frequency of disease caused by anaerobic organisms of the Clostridium group in the highlands and, also, on the lack of incidence of the anthrax bacillus in the

same regions, despite its widespread occurrence in the rest of the country. Speculations have been made on the possible influence of high-altitude, low-oxygen tension which might favor Clostridium propagation but prevent anthrax bacilli.

It is a well-known fact that livestock originating in the lowlands and taken to high altitudes would very often develop brisket disease. This disease was described for the first time in Colorado in 1915 (11). Since that time, the disease has been reported in several South American countries. It has been known in Peru for many years, where workers confirmed previous indications, that brisket disease was caused by a lack of acclimatization to high altitudes. Lesions observed on the adrenal glands of both cattle and sheep have been interpreted as evidence of an adaptation syndrome that had reached the phase of exhaustion.

The difficulties of successfully rearing livestock in the highlands have existed since ancient times. These difficulties and the problem of mobilizing men and animals in different altitudes were faced and overcome by the ancient conquering tribes.

According to an early Spanish priest (6), the Spanish settlers of Potosi, 14,000 feet above sea level in Bolivia, did not reproduce until some 50 years after the city was founded. However, the natives reproduced 100 percent. The original capital of Peru was inland at an altitude of 13,000 feet; however, it was moved to sea level at Lima in 1639 because horses, chickens, and hogs were unable to reproduce.

An analysis of the effects of altitude on man and the use of such analyses for interpreting the effects on animals make an interesting study.

Since prehistoric times, congenital or acquired acclimatization has permitted normal development of life, even in the high altitudes of the Andes. At present, more than 12 million people live there in normal conditions at 85 mm. oxygen tension instead of 150 mm., which is the tension at sea level. In considering the permanent biological changes that are caused by ancestral acclimatization to chronic anoxia, we must realize that the inhabitants of the high Andes belong to a climato-physiological variety of the human race, which is a decisive factor in determining their attitude and sociological behavior.

One writer (7) has said, "One should rather speak of adaptation for the recent arrivals and of acclimatization as regards those native to the place. The indigenous races become acclimatized to the high altitudes through an age-long process. One reaches a state of acquired acclimatization by passing first through an adaptive period during which the organism overcomes the 'climatic aggression' of the elevation. Climatic aggression is defined as the harmful effect of the Andean 'air-temper' on the coastal dweller and, contrariwise, of the coastal 'air-temper' on the uplander. This factor was considered in the Inca social legislation and used in their tactics of war."

Adaptation is a form of altitude sickness. During this period, the organism overcomes the "climatic aggression" of the elevation. The mechanism of adaptation comprises the "Soroche" or mountain sickness. In its acute stage, it can be felt and seen. In its subacute stage, it is more recognizable to the clinical observer than to the patient. Adaptation, then, is a form of altitude sickness, and acclimatization is the cure. Those who are unfitted for acclimatization are eliminated by a process of natural selection (chronic mountain sickness), which is either visible or inapparent. A significant index of acclimatization is fertility.

If athletic prowess is obtained by training the body to an oxygen deficiency, then the permanent oxygen deficiency of high altitudes is necessarily conducive to athletic prowess. Hence, increment to fatigue must be an expression of the organic law of the high altitudes.

Previous research (2) revealed that a man at 14,900 feet above sea level withstood longer periods of work and used less oxygen in relation to body weight than a workman at low altitudes. In other words, he used oxygen with more efficiency. In doing muscular work, a man living at this altitude (Morococha, Peru) produced less lactic acid than a man living at sea level in Lima who did an equal amount of work.

The man of the highlands, regarding respiratory functions of the blood, has 2 liters more of blood. He has almost double the concentration of hemoglobin, bilirubin, and pyruvic acid. His heart is the heart of an athlete (8), larger than normal because of changes in its right side (2).

Some of the differences found by Hurtado (2) between the man of Morococha (barometric pressure 444 mm., oxygen tension 84 mm.) and the man of Lima (barometric pressure 760 mm., oxygen tension 147 mm.) can be observed in table 1.

Acid-base equilibrium.--People in Morococha have greater carbon dioxide tension and, therefore, less carbonic acid in arterial blood than those in lower altitudes. As compensation, a lesser amount of bicarbonate is present and the pH is maintained within normal limits.

Hematologic studies of sheep (1) showed that the red blood cell count, the hemoglobin content per 100 cc. of blood, the mean globular volume, and the hematocrit values were higher for sheep at the 12,000-feet altitude than for sheep at sea level. The mean globular hemoglobin and the mean hemoglobin concentration (percentage) were the reverse.

According to Rotta and his colleagues (9), "A moderate, but significant degree of pulmonary hypertension has been found in men living at high altitudes (14,900 feet), this condition being more accentuated in the permanent than in the temporary residents and most marked in the cases of chronic mountain sickness. The probable pathogenesis of this condition has been discussed from the degree of anoxia observed and the values corresponding to the total

and pulmonary blood volume and the cardiac output." (These last cases had been reported earlier [7] and usually correspond to native residents who show clinical symptoms of having lost their natural acclimatization to the environment where they live.)

According to Hurtado (2), who has studied the physiopathology, such cases are basically characterized by an accentuation of the polycythemia and the degree of arterial unsaturation. The cause of the latter, at least in some cases, is a relative hypoventilation if the values are compared to the ventilation values shown by normal men in the same altitude. The relative hypoventilation mentioned before would be due to decreased sensibility of the respiratory center to the chemical stimuli, which regulate this function. In the cases studied by Rotta (9) and others, there was a marked degree of anoxemia associated with an increased circulating and pulmonary blood volume. There was also an abnormal increase of heart volume output. Several factors could contribute to the elevation of pulmonary pressure.

The effect of high altitude on animal reproduction has been studied by several authors. A reference has been made before about impairment of reproduction of the Spanish conquerors at Potosi in Bolivia (6).

The fertility of cats and rabbits at altitudes of 12,000 feet was studied by Monge and Mori-Chavez (5). They noticed an inhibition of spermatogenesis although not in every animal. Regarding cats, they found that the cells of seminiferous tubules were replaced by Sertoli's syncytium, a mass of thick, granular protoplasm. There was an increase of Leydig's cells, as in the resting period of certain animals that exhibit periodical spermatogenesis, and there were no spermatogoniae. Monge and Mori-Chavez also found an inhibition of spermatogenesis in some rabbits but not in all. Only the phase of spermatogoniae was reached and, among the germinal cells, a great number of Sertoli's syncytium were noticeable.

Fertility studies carried out on rams taken from sea level to higher altitudes showed that only 50 percent were capable of reproducing during the first year and 70 percent, after 2 or 3 years. Thirty percent were infertile. However, native sheep that have been acclimatized since colonial times are almost 100 percent fertile (6).

Studies were made of semen to determine the cause of low fertility of rams taken to high altitudes. This low fertility was found to be caused by several factors. The deviation of the semen pH was greater than at sea level. Although the number of spermatozoids was high, there were numerous young forms, spermatozoid motility was decreased, and leukocytes and erythrocytes as well as epithelial cells were often found. Only 26 percent of 58 rams examined were suitable for reproduction. The reduced birth rate of sheep at high altitudes is blamed on a diminished ability of fecundation of seman (10). Azoospermia has been observed in sheep having chronic mountain sickness after being taken from sea level to an altitude of 10,000 feet. Recovery after 5 months has been observed in some instances but not in others (4).

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TABLE 1.--Physiological features of a man living at sea level and of one living at a high altitude

Feature	Values for man living at--	
	sea level in Lima, Peru 1/	14,900 feet altitude in Morococha, Peru 2/
Ratio of weight/height	37.0	32.6
Air volume in lungs --- liters	1.5	2.0
Air volume in lungs per minute in resting stage --- liters	8.0	10.4
Oxygen pressure:		
Tracheal mm. mercury	147	84
Alveolar mm. mercury	98	50
Capillary mm. mercury	70	38
Oxygen saturation of hemoglobin --- percent	96-98	80
Hematology:		
Red blood cell count --- millions	5.12	6.42
Hemoglobin -- grams per 100 cc. blood	15.60	20.20
Diameter peripheral erythrocytes -- microns	7.48	7.74
Volume of blood -- cc. per kg. of body weight	85.2	105.2
Blood plasma -- cc. per kg. of body weight	46.5	37.6
Volume of erythrocytes -- cc. per kg. of body weight	38.5	67.0
Arterial blood -- Vols. CO_2 per 100 cc. of blood	45.7	34.7

1/ Barometric pressure 760 mm., oxygen tension 147 mm.

2/ Barometric pressure 444 mm., oxygen tension 84 mm.

SECTION III - METHODS OF STUDYING HOST REACTIONS TO INFECTION

Martin Roepke, Ph. D., discipline leader in biochemistry and biophysics, National Animal Disease Laboratory, presiding 1/

HISTOCHEMISTRY OF HOST RESPONSE TO INFECTIONS

Max Wachstein, M. D. 2/

Histochemical methods have so far been applied only irregularly to the study of infectious diseases. Perhaps it would be useful to point out first the technical problems involved in applying such methods.

Histochemistry and cytochemistry are concerned with the characterization and topographical localization of chemical components in cells and tissues. Staining methods suitable for this purpose have been in use for some time. They have been employed mainly, however, for identifying cellular and intracellular structure, which can be observed in tissue preparations stained with routine techniques. Some granules, for instance, that appear yellow to brown in liver sections stained with hematoxylin-eosin give a positive reaction for iron. Thus, they can be identified as hemosiderin. In the case of cytoplasmic vacuoles that remain unstained in paraffin-embedded liver sections, appropriate staining of frozen sections will enable one to conclude that these vacuoles correspond to fat droplets, which have been dissolved during the preparation of the paraffin section. The rapid development, however, of histochemistry in the last decade has been responsible for the emergence of many new techniques, which make possible the localization and identification of chemical substances and enzymatic activities that have been previously recognized only in tissue extracts.

With most organic and inorganic substances, the method of tissue preparation does not pose great difficulties; however, the situation is more complicated in the case of enzymes. It seems, therefore, pertinent to discuss briefly the problems with which one is confronted in this type of work. These are concerned mainly with two aspects: (1) the method of tissue preparation and (2) the visualization of enzymatic activities in tissue sections.

1/ The following paper was presented at the symposium but was not available for publication: Bio-electrical Potentials of the Alimentary Canal, by Charles F. Code, M. D., the Mayo Clinic, Rochester, Minn.

2/ Departments of Pathology, St. Catherine's Hospital, Brooklyn, N. Y., and the Beth Israel Hospital, Passaic, N. J. The work reported here was supported by Grant A-688-C, National Institutes of Health, U.S. Public Health Service.

As far as preparation of tissues is concerned, either fixed or unfixed material may be used. Frozen sections can be cut directly from fresh, unfixed tissues on a freezing microtome or in the linderstrom-Lang type cryostat at low temperature. One can also cut frozen sections from tissue blocks fixed in cold neutral formalin or acetone. One may also prepare paraffin sections from tissue fixed in cold acetone followed by routine dehydration techniques, or from frozen dried material, or from tissue that has been subjected to freeze substitution. For some enzymes, unfixed frozen sections must be used, since any type of fixation renders the tissue unreactive.

In order to demonstrate products formed by enzymatic activity, they must be precipitated at the exact place of origin. The newly formed precipitate must be easily convertible into a colored product. For this purpose, certain products of enzymatic hydrolysis can be converted into lead, cobalt, or copper salts, which can be visualized as dark sulfides, or they can be coupled with diazonium salts to form brilliant azo dyes. Another method for the visualization of certain enzymatic activities makes use of the formation of insoluble colored compounds by the action of oxidatives or reducing enzymes or by the formation of insoluble substances that can be visualized by a special staining reaction.

A very helpful approach in the critical evaluation of histochemistry of tissues or cells is to compare results of histochemical staining reactions with those of fractionation of tissue homogenates. Thus, the localization of certain oxidative enzymes in mitochondria has been established by both approaches. On the other hand, inconsistencies revealed by these two techniques must be resulved by further studies. One point needs particular emphasis -- the method of tissue preparation often affects the obtained staining results. This is explainable in part, at least, by the depressing effect of fixatives or chemicals or both used in the tissue preparation. Histochemical methods permit the localization of chemical substances or enzymes into single cells, but most cells in such a section may not react. This localization of substances indicates the usefulness of and often the superiority of histological methods over biochemical methods, which necessitate destruction of anatomical structures and subsequent dilution of the substance in question, even if present in great concentration in single cells.

Chemical substances can be localized in cells or groups of cells with histochemical or staining methods on what one might call a histological level. This can be accomplished with many different techniques.

As an example, figure 1 demonstrates the localization of glucose-6-phosphatase in the rat kidney. Enzymatic activity is almost limited to the proximal portions of the proximal convoluted tubules, whereas nonspecific esterase is active to a different degree in most segments of the nephron (fig. 2).

Many techniques have now progressed to a stage where not only cellular but also intracellular localization is possible. Differential centrifugation of tissue homogenates shows that certain enzymes are found in mitochondria, whereas others, mainly the hydrolytic enzymes, are present in somewhat small particles, called lysosomes or phagosomes. Certain enzymes are bound to the endoplasmic reticulum (microsomes) and appear in the supernatant fluid.

For the demonstration of oxidative enzymes in mitochondria, tetrazolium salts are most valuable. They form soluble, colorless substances in the oxidized state. However, they become insoluble and colored when they have accepted hydrogen by the action of various hydrogenases. Thus, one can demonstrate different dehydrogenases according to the system in which these indicators are used. Some of these dehydrogenases need DPN or TPN (diphosphopyridine nucleotide or triphosphopyridine nucleotide) for their action whereas others, such as succinic dehydrogenase, do not require either.

Figure 3 shows typical mitochondrial localization in proximal convoluted tubules of a rat kidney in a section stained for the presence of DPNH diaphorase (tetrazolium reductase). Another enzyme that can be localized in mitochondria is ATPase (adenosine triphosphatase) (75). 3/ Figure 4 reveals distinct ATPase activity in the mitochondria of a rat liver.

Phagosomes (67, 68) or lysosomes (31) are granular bodies, which were first discovered in differential centrifugation studies of cell homogenates. These granular bodies can be best demonstrated histochemically with the techniques for acid phosphatase (fig. 5). Microsomal enzymes give a diffuse cytoplasmic reaction. In certain instances, the use of inhibitors permits a histochemical differentiation of microsomal and lysosomal localization. In the case of nonspecific esterase and thioacetic acid esterase, certain inhibitors (organophosphorous compounds) suppress the diffuse cytoplasmic reaction and thus lysosomal activity, which is resistant to the inhibitors (80). This is made apparent in figs. 6 and 7.

In addition to distinctly localized intracellular activity, certain special structures give distinct reactions. Examples are motor endplates stained with certain esterase techniques (fig. 8) or brush borders of the proximal convoluted tubules in kidney sections prepared for the demonstration of alkaline phosphatase (fig. 9). In spite of considerable technical difficulties, the localization of chemical substances and enzymatic activities has become possible with the electron microscope. This is evident in the demonstration of ATPase in bile canaliculi (35), or of peroxidase in leukocytes (52). Correlated morphological and chemical

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3/ Numbers in parentheses refer to Literature Cited at the end of this paper.

studies in tumor cytochemistry have been reported (57). Histochemical methods have been applied not only to the study of normal tissues and cells but also under abnormal ones. To the best of my knowledge, no effort has been made so far to apply histochemistry in any systematic way to the study of infectious processes except for the very considerable work that has already been done in virus infected cells. The present discussion will consider phenomena commonly associated with infections, particularly those related to inflammation and wound healing.

Virus Infections

The biochemistry of virus-infected cells has received much attention, and results have been recently summarized by S. Cohen (21). In spite of much research, the biochemical behavior of the infected animal cells, the unit of virus disease, is not clarified as yet. The synthesis of nucleic acids has been studied with conventional and isotope techniques, and the behavior of respiration of virus-infected cells has been scrutinized. Studies of enzyme changes with biochemical methods have led to interesting observations. An increase of xanthine oxidase (7, 63) and a marked drop in various other enzymes, particularly acid and alkaline phosphatase and nucleotidase (47, 48), have been found. Bachtoldt and others (3), however, detected a decrease in alkaline phosphatase and ATPase only when cytopathogenicity became pronounced, whereas glucose-6-phosphatase even increased under certain conditions.

To the best of our knowledge, histochemical enzymatic studies have, so far, not been carried out in infected tissue cultures, although tissue cultures of normal and cancerous cells have been repeatedly investigated with these techniques (73). It is, however, of interest that biochemical and histochemical changes following dissection of peripheral nerves have been found as early as 1945 in ganglion cells that initiated the alterations in nerve cells and occur in infections with poliomyelitis virus (10, 45). Under these circumstances, chromatolysis of Nissel substance occurs. Succinic dehydrogenase increases when assayed biochemically, and there is a marked enhancement also in the intensity of the histochemical staining reaction for acid phosphatase.

In contrast to the paucity of investigations using enzymatic staining reactions, considerable work has been done with regard to the demonstration of nucleic acid and protein substances. In addition to the classical methods for the demonstration of RNA (ribonucleic acid) and DNA (deoxyribonucleic acid) -- basophilic dyes and prevention of the staining reaction by ribonuclease in the case of RNA, and the Feulgen reaction in the case of DNA -- fluorochroming with acridine orange has been used also (2). Protein constituents have been demonstrated with a number of techniques, including those for the demonstration of specific amino acids -- tyrosine, tryptophane, and basic amino groups. A complete review of the results with these staining techniques in various virus infections is not intended here. Only a few examples can be given.

Negri bodies, which are of diagnostic significance in the recognition of rabies, not only contain DNA but also give positive reactions for proteins, various amino acids, and masked iron, but do not react for alkaline phosphatase (55), in contrast to earlier reported findings (86). In an exemplary paper, Bloch and coworkers (12) correlated electron microscopic and histochemical findings in their study of adenovirus infected Hela cells and found that intranuclear crystalline aggregates seen with the electron microscope contained DNA and were, therefore, assumed to be a constituent of the virus particle. These particles developed from a Feulgen negative matrix. In adenovirus type 5, crystals were found not to contain DNA or RNA but to give a number of protein reactions including those for aromatic amino acids, tyrosine, and basic amino groups (41, 49, 54).

Boyer and others (16) detected similar crystals in adenovirus types 3, 4, and 7, which, however, were Feulgen-positive and were considered to be virus-containing as evidenced also by positive immunofluorescent staining. Using the acridine orange technique, Armstrong and Niven (2) examined vaccinia and ectromelia-infected mice and found greenish-yellow, DNA-containing bodies in liver cells, which showed no other microscopic abnormalities. In the liver of mice infected with hepatitis virus, the cytoplasm of hepatic cells contained intensely red, fluorescent RNA-containing inclusion bodies. With a similar technique, Starr and others (65) examined tissue cultures of human cells infected with psittacosis virus. In response to infection with the virus, the whole cytoplasm contributed a matrix, which stained for RNA and particles revealing DNA.

Hepatitis induced by equine abortion virus in hamsters was investigated by Randall and coworkers (39, 60). As early as 6 hours following infection, a striking intranuclear accumulation of material giving a positive reaction for basic proteins was found at a time when the Feulgen reaction in this material was still negative. In the case of the viral papilloma of the skin, inclusion bodies likewise contain not only DNA but also a relatively basic protein, which stains with the alkaline fast green method for histones (11). The cytoplasmic inclusions of vaccinia lesions contain abundant protein stainable by the OTA (oxidized tannin-azo) method and also by the tetrazo method of Danielli (27).

In the liver of rhesus monkeys infected with yellow fever virus, Baercroft (4) detected an increased reaction for RNA, first to occur in the nucleolus and later also in the cytoplasm. DNA increased slightly in the nucleus and collected on the nuclear membranes. The typical Torres inclusion bodies gave a positive reaction for histones and for a variety of amino acids but they were free of nucleic acids, carbohydrates, and fats. The inclusions apparently did not contain virus. Liver cells in addition revealed degenerative changes. In such cells, there was loss of glycogen, increase in neutral fats, and finally loss of RNA.

Similarly in vaccinia-infected cells in young rabbit skin, protein stains demonstrate, in addition to a positive reaction in the inclusion bodies,

profound loss of cytoplasmic protein or coagulative necrosis with retention of protein (29). These histochemical changes are obviously nonspecific and represent changes occurring in cell degeneration and necrosis.

Direct Effect of Infections and Toxins in Histochemical Staining Reactions in Host Tissues

In view of the paucity of significant anatomical findings in death due to bacterial toxins, several investigators have applied histochemical techniques for the study of tissues of animals dying because of bacterial toxins. Wattenberg (82) studied a number of tissue enzymes in mice given lethal doses of murine typhus toxin, but he found no significant alterations. On the other hand, profound changes of alkaline phosphatase in the kidney were described by Berg and Levinson (9) following the administration of a lethal dose of Clostridium perfringens toxin in dogs. Gradual shock developed in 4 hours. At that time, the kidneys revealed a striking inactivation of alkaline phosphatase activity in proximal convoluted tubules in spite of their frequently normal microscopic appearance in routinely stained sections. The administration of dextrose and, to a lesser degree, of several amino acids, although not preventing the fall in blood pressure and death, normalized the renal phosphatase reaction.

In guinea pigs dying from anthrax infections, marked cellular degeneration and necrosis of the proximal convoluted tubules of the kidney are associated with a concomitant diminution in alkaline phosphatase (61). A segmental decrease in renal alkaline phosphatase without significant changes in routinely stained H. and E. sections was observed by Spink and Landeryou (64) in shock produced by Escherichia coli endotoxins. Kidney damage may be directly due to the effect of bacterial toxins or at least partially to vasoconstriction. Indeed Stoner (66) found that shock induced by ischemia of the limbs led to a varying degree of histochemical diminution of renal alkaline phosphatase. It should, however, be mentioned that neither complete ligation of all renal vessels nor severe tubular necrosis induced by various poisons leads to such complete inactivation of renal alkaline phosphatase (78) within 24 hours as has been described to occur following administration of C. perfringens toxin within 4 to 5 hours.

When rats were infected with Salmonella typhimurium, there occurred an increase in stainable acid phosphatase and esterase, not only in Kupffer's cells (see later), but also in the cytoplasm of parenchymal cells, mostly peribiliary in location (81). Enzymes localized in mitochondria such as succinic dehydrogenase, adenosine triphosphatase, and DPN diaphorase were not influenced. In bile canaliculi, ATPase activity was often markedly decreased (figs. 10 and 11), staining for 5 nucleotidase was not influenced, and alkaline phosphatase was even increased (fig. 12). In contrast to the decrease of ATPase activity in bile canaliculi, sinusoids revealed increased ATPase activity (fig. 11). The sinusoids did not reveal any evidence of alkaline phosphatase. The mouse, however, shows an irregular staining reaction in the sinusoids, and this is markedly increased in infected animals (74, 81).

In the livers of animals infected with S. typhimurium, infiltrates of inflammatory cells and focal necrosis were regularly seen. In necrotic cells, all staining reactions became negative. The cellular infiltrates, which are composed of various types of mononuclear cells, revealed in some unidentified cells a positive reaction for alkaline phosphatase. Non-mitochondrial ATPase was reactive in most of these inflammatory cells, but esterase and mitochondrial enzymes showed only a weak reaction.

Some information has been accumulated concerning the effect of nonbacterial infectious agents. Acid and alkaline phosphatase were studied in muscles of rats infested with Trichinella spiralis (18). Basophilic granular degeneration occurring 1 to 2 days after invasion of muscle fibers was thought to be due to increase of cytoplasmic ribonucleic acid. Alkaline phosphatase not normally present became manifest 4 to 5 days after fiber invasion and remained undiminished in the vicinity of the trichinae for many months. Recently it has also been found that aminopeptidase was increased in areas close to the invading trichinae (8). In the livers of mice, increased alkaline phosphatase activity was seen following experimental infestation with Schistosoma mansoni. Phosphatase was prominent at the connective tissue capsule around typical granulomata (30).

The influence of parasitic infestation on liver histochemistry was investigated by Brand and Mercado (17, 51). In contrast to results with fasting normal control rats, the feeding of fructose and glucose to rats infected with Plasmodium (Berghi) and other parasites led only to a spotty periportal deposition of glycogen. Meticortelone (R), however, corrects this metabolic defect to some degree. There is also centrolobular deposition of lipids in rats infected with Plasmodium (Berghi). In rhesus monkeys infected with Plasmodium knowlesi, little stainable liver glycogen is found, even after infusion of glucose. Glycogen was, however, deposited after administration of glucose and adrenal hormones (28).

In guinea pigs infected with tuberculosis, alkaline phosphatase of the adrenal cortex is normal in the zona glomerulosa, but markedly reduced in the zona fasciulata and reticularis (46). Brand and Mercado observed lipid depletion in the adrenal cortex of rats infected with Plasmodium (Berghi). This was mitigated by cortisone (17). On the other hand, Devaked and Macgraith (28) found no loss of stainable lipids in the adrenals of rhesus monkeys that were infected with Plasmodium knowlesi.

Acute and Chronic Inflammation

The response of the skin to acute injury was studied by various investigators by excision of segments of skin and underlying muscle. In most instances, no special effort for sterility was made. The ensuing reaction is characterized by the deposition of a ground substance, which stains metachromatically with toluidine blue and gives a marked reaction with Hale's colloidal iron stain for mucopolysaccharides. This early staining reaction disappears as the fibroblasts mature and collagen is laid down (69).

Fell and Danielli (36) were the first to study alkaline phosphatase in wound healing and found in the early phase of acute inflammation a positive reaction in the invading polymorphonuclear leukocytes. Later the regenerating connective tissue becomes strongly positive with fibroblasts, intracellular fibers, and capillaries staining deeply. A relationship of this reaction to vitamin C is indicated by the marked depression in enzyme content of granulation tissue in scorbutic guinea pigs (27). A strong reaction for aminopeptidase in the areas of wound healing is mirrored by an increase in the level of this enzyme in the serum (53). Raekallio (59) found in guinea pigs that leucine aminopeptidase increased as early as 2 hours after wound healing, whereas acid and alkaline phosphatase increased a few hours later. Strong activity of beta-glucuronidase was described also (20). In human gingiva showing acute and chronic nonspecific inflammation, Burstone (19) found marked increase of histochemically demonstrable cytochrome oxidase in inflammatory cells and in the epithelium.

Although many bacterial incitants lead to an acute type of reaction, certain others produce granulomatous lesions. In man, such lesions were studied in skin biopsies (37, 58, 84). In leprosy, nonspecific esterase and acid phosphatase were found in epitheloid cells and in giant and lepra cells; alkaline phosphatase was only in capillary walls and occasionally in the vacuoles of giant cells. Neutral fat was also present in some of the epithelioid cells.

Granulomatous lesions have also been studied in experimental animals. Grogg and Pearse (42) inoculated mice, rats, guinea pigs and rabbits with bovine tuberculosis bacilli, and studied enzymatic and lipid reactions. Acid phosphatase was considered to be the most important enzyme, since it was present in phagocytes and mononuclear cells of animals relatively resistant to tuberculosis (mouse, rat), whereas in the nonresistant guinea pig no intracellular and only slight extracellular staining reactions were seen in caseating tubercules. In confirmation of older observations, sudan-positive droplets were present in macrophages. Treatment with antituberculous drugs did not influence these reactions.

Goessner (40) studied human tuberculosis and found strong esterase and acid phosphatase activity in giant cells. In epithelioid cells, he found esterase was strongly positive, but acid phosphatase was only slight. Alkaline phosphatase occurred in the fibrosing tubercules in the cytoplasm and fibers of fibroblasts and in capillaries. Gedigk and Bontke (38) produced foreign body granulomata in mice and rats by various means and found strong acid phosphatase, beta-glucuronidase, and less consistent esterase activities in phagocytosing cells. Eder (32) extended these investigations to a number of species, including those previously examined by Grogg and Pearse, and found the same species differences in reactivity to nonspecific foreign body granulomas as in tuberculosis.

It would, therefore, appear that a different histochemical response in various species cannot be correlated to differences in host susceptibility to a tuberculous infection but is an expression of considerable species variation <u>per se</u>. This marked difference in histochemical reactivity must

always be considered and great pains must be taken not to generalize results gained in one species without adequate controls. In addition to hydrolytic enzymes, foreign body granulomata also exhibit strong staining reactions for various oxidative enzymes (5).

Reticuloendothelial System and Myeloid Cells

The RE (reticuloendothelial) system is considered to be of great significance in infectious processes. Cells belonging to this system have been investigated by morphologists, immunologists, and biochemists alike. Histochemically, such cells can be either studied in tissue sections, or single cells can be obtained for study as, for example, monocytic cells harvested from the peritoneal cavity following the injection of heavy mineral oil. Some of the cells that have been considered in the preceding paragraph dealing with chronic inflammation are probably of reticuloendothelial origin.

Biochemical investigations have been undertaken in order to study alterations of RE cells under various stimuli including infections. In view of the as yet unexplained resistance of certain species to a given infection, differences in the biochemical reactivity of susceptible and resistant animals have provoked considerable interest. Thus, Allison and others (1) studied monocytes from the peritoneum of rabbits genetically susceptible and resistant to tuberculosis for their ability to metabolize certain substrates. They found that the cells of resistant animals exhibited greater activity of various dehydrogenases and of acid phosphatase than those of susceptible animals.

Histochemically Thorbecke and others (70) found monocytes from the peritoneal cavity of mice infected with BCG to contain considerable more acid phosphatase than those from normal controls. The acute effect of phagocytosis on monocytic cells from the rabbit peritoneum was studied by Danneberg and others (25, 26). No changes were seen in the amount of acid phosphatase, alkaline phosphatase, nonspecific esterase, and succinic dehydrogenase within 30 to 60 minutes. These negative results may, however, be due to short duration of the experiment, since in tissue cultures macrophages that had ingested red blood cells exhibited increased acid phosphatase activity (34). Hosoda and Nakamura (43) used loose subcutaneous connective tissue spreads and found marked increase in acid phosphatase, ATPase, beta-glucuronidase, and leucine aminopeptidase in macrophages following various irritants including bacterial ones.

Considerable increase in vitamin C in the macrophages of the lung, particularly in experimental pneumonias, has been described as early as 1938 (50, 71). More recently vitamin C was also found in some cells in infiltrates of typhoid fever (56).

Spreads from peritoneum were used by Eder and Schauer (33) for the study of mast cells. These cells contain within the typical metachromatic granules various enzymes, particularly ATPase, aminopeptidase, acid phosphatase,

and beta-glucuronidase. Following the administration of histamine liberators, the granules are dispersed and lose their enzymes. Acute local inflammation induced by putting croton oil or streptolysin or bacteria on the exposed peritoneum led to a similar degranulization and dispersion of histochemically demonstrable enzymes.

That the reticuloendothelial system in toto can be influenced by various stimuli is well known. Its functional capacity can be measured in various ways in the intact animal, for example, by the rate of clearance of intravenously injected carbon particles. Such an increase in clearance can be demonstrated after experimental infection (13, 14). Increase in enzymatic activity of Kupffer's cells following various stimuli has been observed by various investigators (51, 79). Howard (44) studied Kupffer's cells of the mouse liver after administration of bacterial lipopolysaccharides and found a distinct increase of acid phosphatase activity. A single injection of typhoid vaccine led to an increased amount of acid phosphatase containing reticuloendothelial cells and to a decreased number in the spleen (6). Increases in the number and intensity of the reaction were also found in the liver of mice following inoculation with BCG (70) (bacillus Calmette-Guerin). A varying increase not only of acid phosphatase but also of esterase activity is observed in Kupffer's cells of the liver of rats infected with S. typhimurium (81) (figs. 15 and 16).

Böhme and others (15) applied the acid phosphatase test to the study of S. typhimurium infections in genetically resistant and susceptible mice. There was a marked increase in positive-reacting cells in both strains. However, in agreement with similar results with the carbon clearance test (14), the response of reticuloendothelial cells could not be correlated with the outcome of the infection, which terminated in death in all susceptible animals and in a high survival rate in the resistant ones.

Polymorphonuclear leukocytes have been the subject of much biochemical investigation and have also been studied extensively by histochemical methods. Shortly after the introduction of the Gomori-Takamatsu technique for the demonstration of alkaline phosphatase, a strong reaction in inflammatory foci was seen in tissue sections in areas of acute inflammation (36). Polymorphonuclear leukocytes in human blood smears and bone marrow cells show a striking increase in alkaline phosphatase activity in infection (72).

Polymorphonuclear leukocytes have the ability of phagocystosis and thus play a great role in the reaction to infection. Phagocytosis is an energy-requiring process, in which glycolysis serves as the most important source of energy. An initial period of glycogenolysis is followed by enhanced glycogen synthesis (24).

Cohn and Hirsch (22, 23) fractionated rabbit polymorphonuclear leukocytes and isolated the specific granules. These contain all acid and alkaline phosphatase, nucleotidase, ribonuclease, desoxyribonuclease, and beta-glucuronidase and about 50 percent of cathepsin present in the leukocytes.

Thus, these granules seem to have similarity to De Duve's lysosomes. During phagocytosis of various micro-organisms or yeast, marked reduction in the number of cytoplasmic granules takes place within 30 to 60 minutes. The granular enzymes are released in the cytoplasm, but the total amount of enzymatic activity of the white cells is not changed. However, Danneberg and others (25, 26) found no change in various histochemical enzyme reactions under similar conditions. The difference in these findings is as yet unexplained and needs urgent reinvestigation. It should, however, be stated that histochemical localization in granules of leukocytes has so far been accomplished only in some instances, particularly ATPase (83), acid phosphatase (62) and thioacetic acid esterase (74).

In addition to the reactions mentioned in the previous paragraphs, the application of antigenic substances, including bacterial ones, leads not only to activation of macrophages but also to a marked stimulation of primitive reticular cells. These can be well studied in the rat spleen (85). Such antigen-forming activated cells are characterized by the abundant pyrinophilic cytoplasm indicating the presence of increased amounts of RNA. The appearance of such cells under various conditions of antigenic stimulation has been described in various locations.

Concluding Remarks

This incomplete presentation brings out the fact that histochemical techniques have come into use in the study of infections. In general, such investigations have so far been carried out in a more systematic way only in virus-infected cells. However, it should be pointed out that histochemistry has already found much broader applications in the study of disease states, which must be considered to have been initiated by infectious processes, for instance in acute glomerulonephritis, with all the detrimental consequences in progressive cases (76, 77).

A question that is of paramount interest concerns the specificity of tissue reactions induced by infectious agents. The evidence at hand would indicate that, in general, damaged tissue cells will react in a similar fashion regardless of the injurious agent. Baercroft (4) pointed out that many of the histochemical changes that occur in degenerating liver cells infected with yellow fever virus must be considered as nonspecific. We have noticed that necrotic cells in the rat liver show similar staining reactions regardless of whether cellular necrosis follows bacterial infections or chemical injury. The activation of sinusoidal epithelium and reticuloendothelial cells as evidenced by the increase in some enzymatic staining reactions is likewise observed not only in infections but also following other stimuli. Nevertheless, much more detailed information will have to be obtained before one can say with certainty whether the response to a given infection is characteristic for a specific agent.

In certain conditions, particularly in the response of cells to virus infection, histochemical techniques have contributed significantly to the elucidation of cellular alterations. In other circumstances, such histo-

chemical techniques have led to the study of new parameters of host response to the infectious agents. Thus, the numerical estimation of acid phosphatase activity in Kupffer's cells can be used for the evaluation of its functional activity. There also exists a fruitful mutual interplay of biochemical and histochemical investigation. The histochemical approach permits the study of single cells in tissue sections in response to infectious agents.

Recent progress in technical methods makes it clear that the electron microscope can be used for the histochemical study of chemically defined substances, for instance glycogen, as well as of enzymatic activities.

In summary, the current techniques and technical problems of histochemical methods have been discussed briefly and some of the applications in the study of infectious processes have been given. It would appear that histochemistry is one of the various, more recent approaches that can be used to convert tissue morphology into a functional discipline for the study of pathological problems, including those dealing with the host response to infection.

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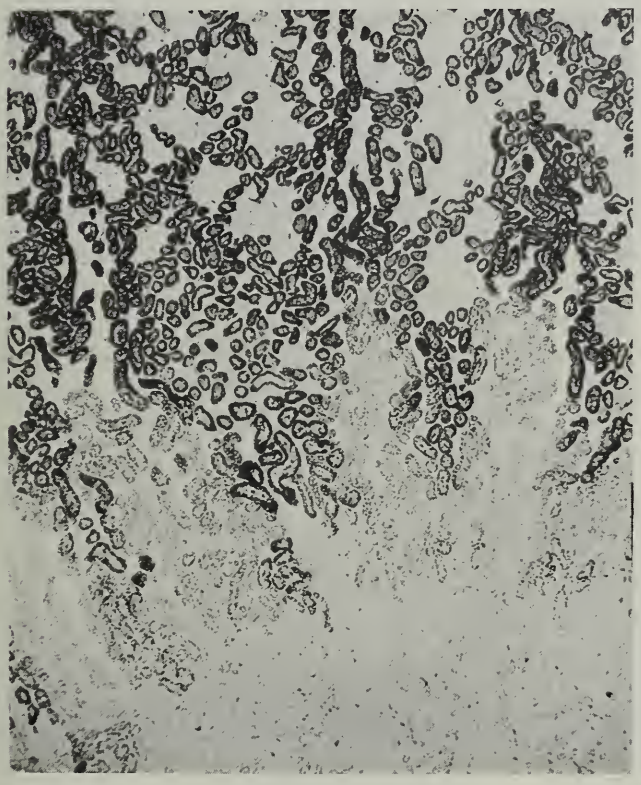

Figure 1.--Unfixed cryostat section of rat kidney stained for glucose-6-phosphatase. The reaction is limited to the proximal portion of the proximal convoluted tubules. Incubation time 15 minutes. X 40.

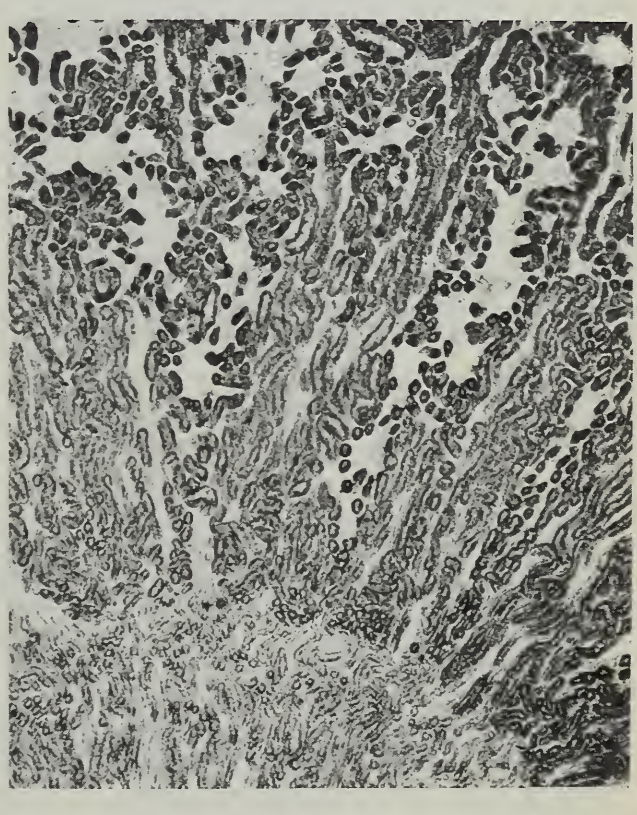

Figure 2.--Frozen section from a cold formalin-fixed rat kidney stained for nonspecific esterase. In contrast to glucose-6-phosphatase in figure 1, there is a much wider distribution of esterase activity in cortical and medullary tubules. (Incubation time unavailable.)X 40.

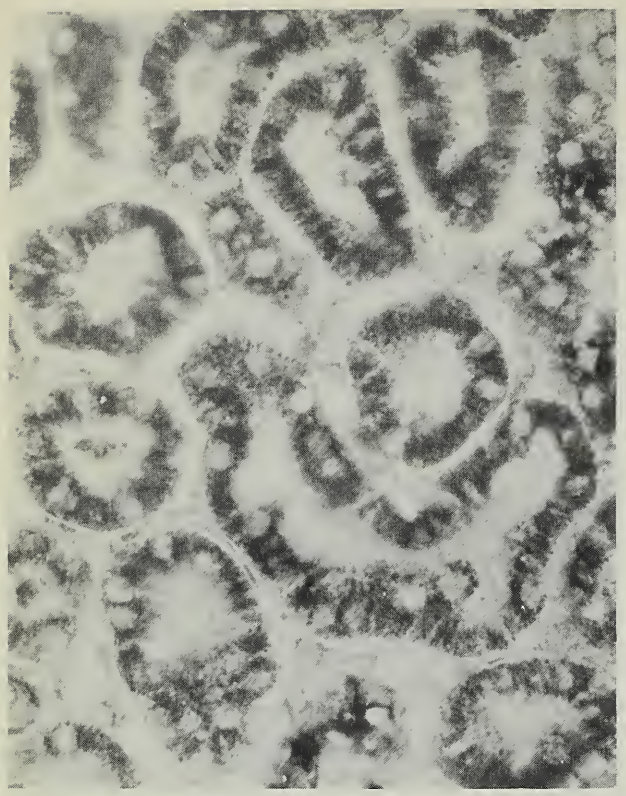

Figure 3.--Frozen section from a formalin-fixed rat kidney stained for DPNH diaphorase. Note perpendicular arrangement of stain deposits indicating predominant localization of enzymatic activity in mitochondria of proximal convoluted tubules. Incubation time 30 minutes. X 640.

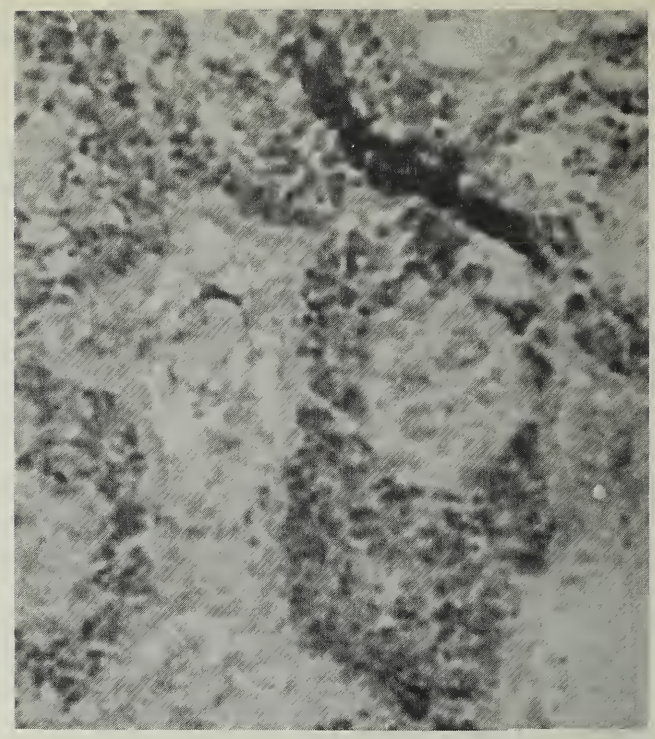

Figure 4.--Cryostat sections from specially fixed rat liver stained for adenosine triphosphatase activity. Stain deposits outline mitochondria in hepatic cells as well as one bile canaliculus. Incubation time 60 minutes. X 2300.

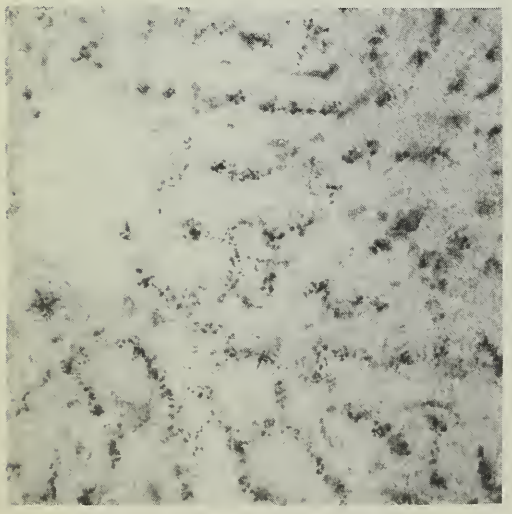

Figure 5.--Frozen section from formalin-fixed rat liver stained for acid phosphatase. Note peribiliary granular deposits corresponding to so-called lysosomes. Incubation time 30 minutes. X 330.

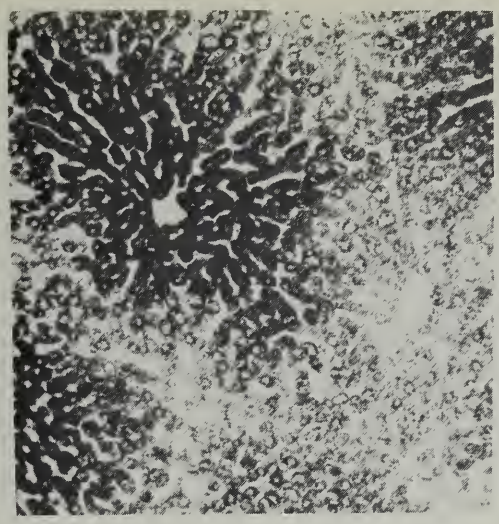

Figure 6.--Frozen section from cold formalin-fixed rat liver stained for thioacetic acid esterase. Activity is strongest in parenchymal cells around central veins. The cytoplasm is diffusely stained. Incubation time 60 minutes. X 100.

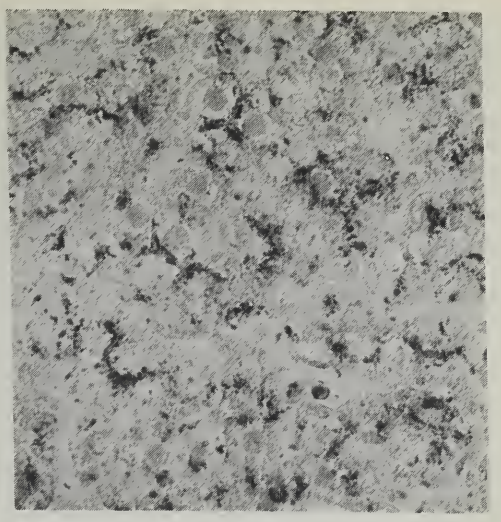

Figure 7.--A section from the same liver as shown in figure 6 stained for thioacetic acid esterase following preincubation for 1 hour in 10^{-5} M E600. Cytoplasmic activity is suppressed and staining is now seen in peribiliary bodies. Incubation time 60 minutes. X 300.

Figure 8.--Frozen section from a cold formalin-fixed rat muscle stained for thioacetic acid esterase. The endplates are clearly visualized. Incubation time 10 minutes. X 80.

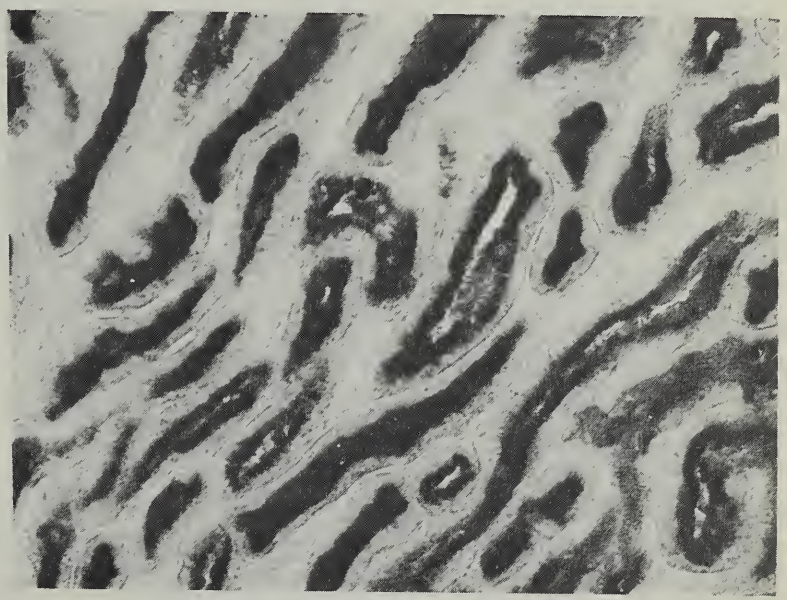

Figure 9.--Frozen section from cold formalin-fixed rat kidney stained for alkaline phosphatase. Note exclusive localization of enzymatic activity in brush borders of proximal convoluted tubules. Incubation time 30 minutes. X 410.

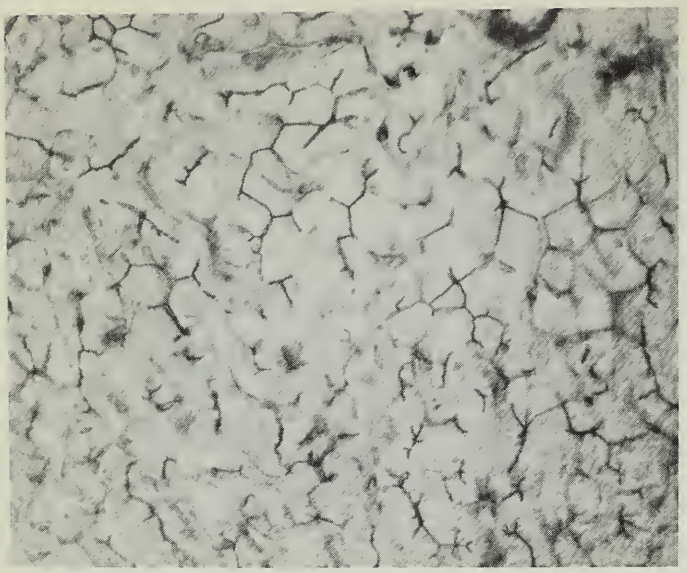

Figure 10.--Frozen section from cold formalin-fixed normal rat liver stained for adenosine triphosphatase. Note prominent staining of bile canaliculi. A central vein in right upper corner also shows marked activity. Sinusoids show only faint irregular staining. Incubation time 10 minutes. X 410.

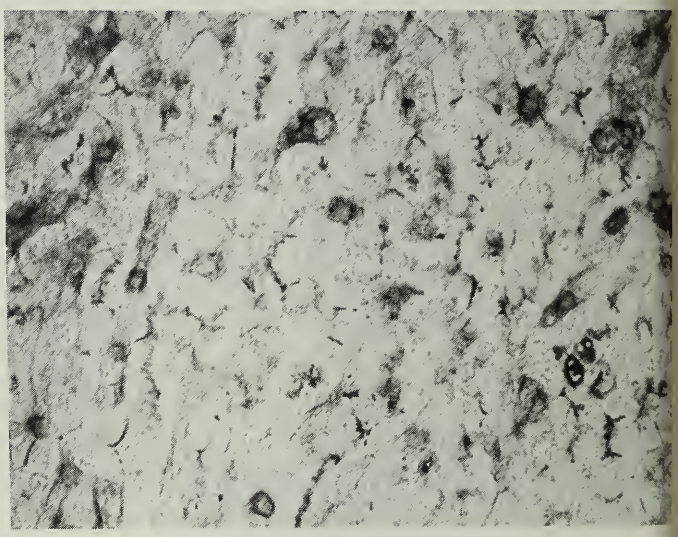

Figure 11.--Frozen section from cold formalin-fixed block of the liver from a rat sacrificed 3 days after infection with Salmonella typhimurium stained for adenosine triphosphatase. Note almost complete suppression of bile canalicular activity and increase in sinusoidal staining. Incubation time 10 minutes. X 410.

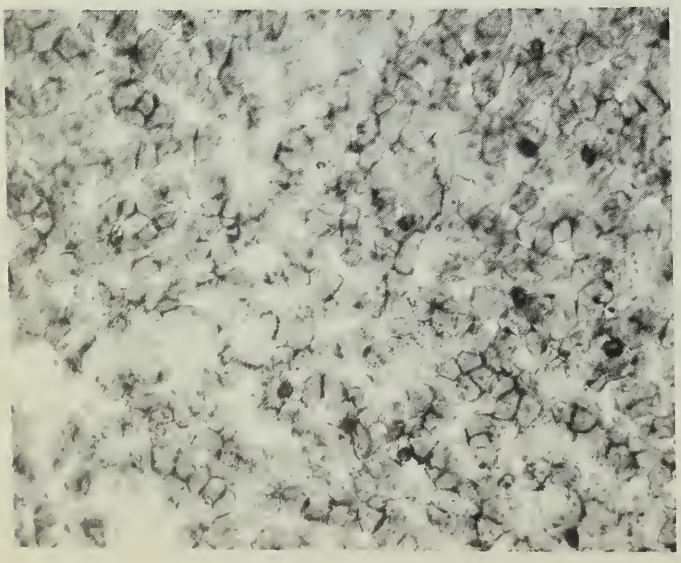

Figure 12.--An adjacent section from the liver shown in figure 11 stained for alkaline phosphatase. Note striking increase in reaction of bile canaliculi. Normal controls show barely visible focal staining in these structures. Incubation time 15 minutes. X 260.

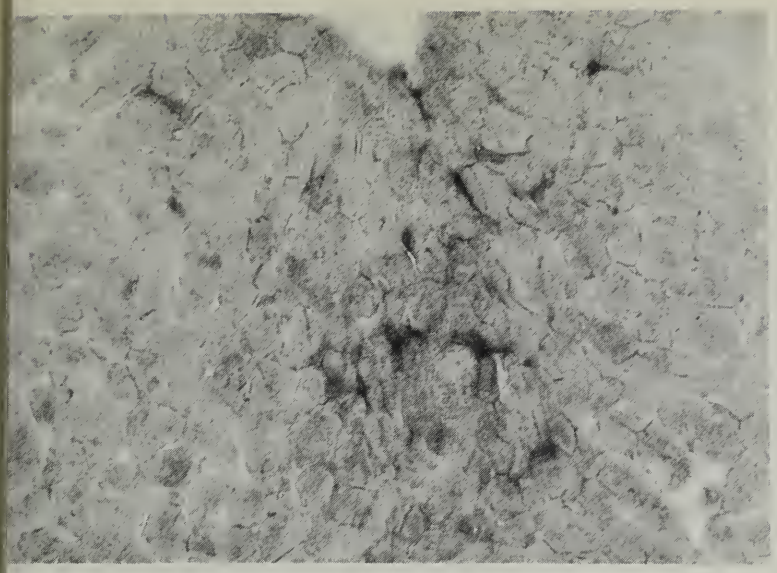

Figure 13.--Frozen section from cold formalin-fixed normal mouse liver stained for alkaline phosphatase. There is slight focal activity in sinusoids. Incubation time 30 minutes. X 330.

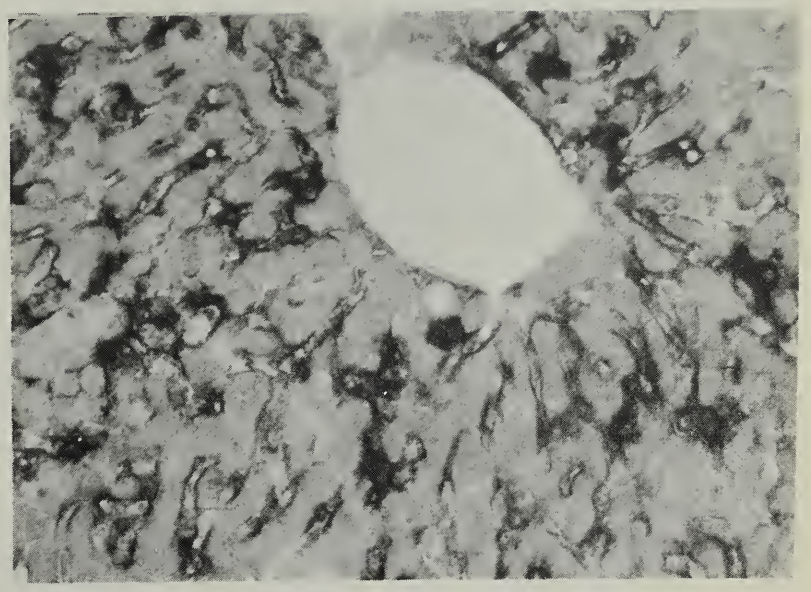

Figure 14.--Frozen section from formalin-fixed liver of a mouse sacrificed 3 days after infection with Salmonella typhimurium. Note the striking increase in sinusoidal activity. Incubation time 30 minutes. X 330.

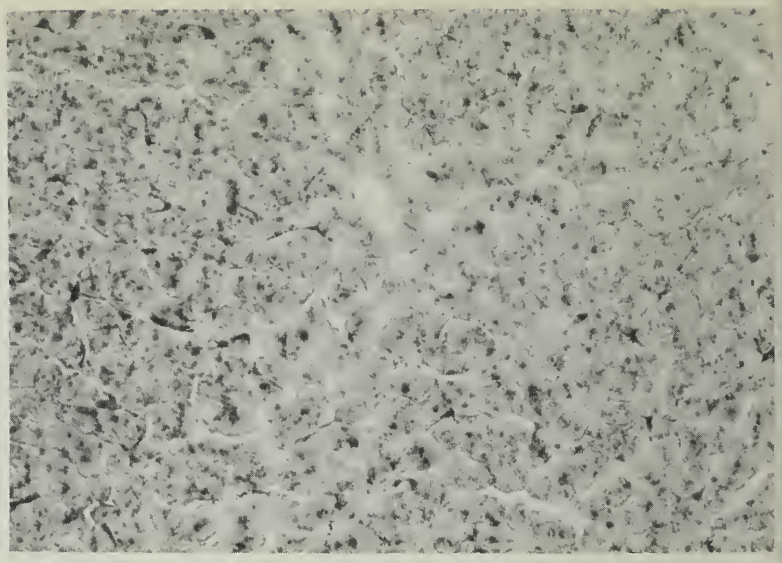

Figure 15.--Frozen section from cold formalin-fixed normal rat liver stained for acid phosphatase. Note activity in occasional Kupffer's cells and barely perceptible staining of peribiliary granules. Incubation time 60 minutes. X 260.

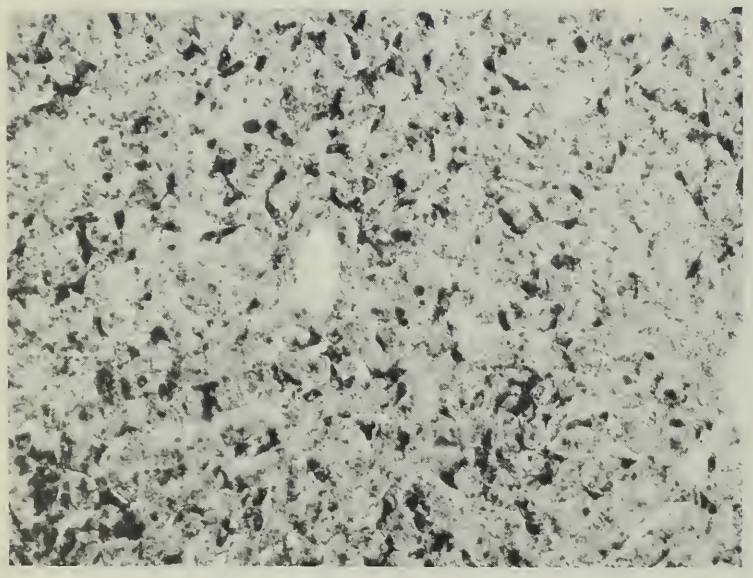

Figure 16.--Frozen section from a formalin-fixed liver from the same mouse shown in figure 14 sacrificed 3 days after infection with Salmonella typhimurium. Note the striking increase of positively reacting Kupffer's cells. Incubation time 60 minutes. X 260.

IMMUNOCHEMICAL METHODS

Keith M. Cowan, Sc. D. 1/

In discussing methods to study host response to infection, it is obvious that the subject of immunology must be considered. Formation of antibodies by host animals to antigenic materials is a response of major interest, and the ability to detect and measure these antibodies, or their corresponding antigens, by relatively simple laboratory techniques is of paramount concern. Detection and measurement of antibodies often provide an index of susceptibility as well as evidence of past or present infection that may, or may not, have been clinically evident. Likewise, antibody-containing serums are of vital importance for identification or typing of various infectious agents. Thus, a great deal of our diagnostic capability depends on immunological reactions. It is probably safe to say that a major part of the animal disease control programs of this country, and perhaps of the world, are primarily based on various manifestations of antigen-antibody interaction. In terms of research on infectious agents, the tools of immunology are indispensable for the study of the host response and the etiological agents.

Serological tests are generally rather simple and are done routinely by many of us attending this symposium. One might wonder why a paper entitled "Serological Methods" was not presented instead of one with the more sophisticated title of "Immunochemical Methods." There is no reason why this could not have been done, but it is hoped that a discussion of immunochemical methods might bring out information pertinent to the interpretation of serological data.

Immunochemistry has been defined as "--the study of the chemistry of antigens and antibodies, and the chemical basis of immunity and resistance to disease." It should be appreciated that not many years ago, the chemical nature of antibody was not known. Although many of us will not claim to be immunochemists, we are delving into immunochemistry any time we refer to the protein nature of antibodies, or the fact that they are gamma or beta globulins.

Concerning the methods of immunochemistry, they are nothing more than the familiar serological methods that have been somewhat refined. Immunochemical methodology was pioneered and developed by Michael Heidelberger and his coworkers at Columbia University. Dr. Heidelberger was a chemist and as such

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1/ Plum Island Animal Disease Laboratory, Animal Disease and Parasite Research Division, Agricultural Research Service, U.S. Department of Agriculture, Greenport, Long Island, N.Y.

reacted rather violently to descriptions of the activity of antiserums or antigen preparations in terms of titer or dilution. He once said, "Immunology and the early immunochemistry as well, staggered under the dictatorship of dilution and the tyranny of titer." In keeping with this attitude, he and a colleague instituted studies on precipitin systems utilizing the precise methods of analytical chemistry. Techniques were developed to measure the antibody content of serum in weight units, usually expressed in terms of antibody nitrogen. Once these quantitative techniques were developed, they were applied to various manifestations of antigen-antibody reactions. As a result, a tremendous amount of fundamental information on the mechanism of immunological reactions became evident. These quantitative studies revealed not only the power of immunological reactions in terms of their sensitivity and specificity but demonstrated their limitations as well.

In terms of sensitivity of the serological reactions performed, these quantitative studies revealed that reactions being observed may be due to amounts of antibody in the range of a millionth to a hundred-millionth of a gram of antibody protein. This is, of course, dependent upon the type of test and the manner in which it is performed. It is the rare serologist that thinks of titration end points in terms of what they mean with respect to the actual quantity of antibody being demonstrated by the particular test performed. If this were done, it might in many instances explain to the investigator why it was not possible to correlate the results from two different types of serological test, for example, complement fixation (CF) titers with virus neutralizing titers.

One major contribution of immunochemical methods applied to routine serological tests has been to point out some of the limitations of the tests, and that judicious interpretation of results is essential. It is not uncommon to hear comments from various workers that CF titers are valueless in their system, or that the system they are working with gives so many peculiar cross-reactions that the serological data are uninterpretable. Such comments are invariably true and usually stem from the fact that we often do not know what is being measured by the test that has been applied. Immunochemical methods have been utilized in an attempt to resolve some of these problems, and although some success has been obtained, there is still much to be done.

It may be of interest to discuss some of the problems encountered and how immunochemical methods have been utilized in an attempt to resolve them, or at least, to explain them. All problems encountered with the different types of serological tests cannot be discussed, so the following comments will be limited to CF and precipitin reactions.

In studying the response of an animal to an infectious agent, or to some other immunizing agent, there often is interest in following the rate of appearance and the level of antibody that is produced. The CF test is often used for this purpose because it is extremely sensitive and adaptable to a

variety of different antigenic substances. When an increase in the C'-fixing antibody titer is observed, this is usually interpreted to mean that an increase in the amount of antibody has occurred. Although this is probably the case in most instances, it is also possible that an increase in titer is not a reflection of an actual increase in the amount of antibody. That has been demonstrated by Wallace and coworkers who have shown that the C'-fixing ability of antibody to pure antigen, such as bovine serum albumin, may vary during the course of immunization (1).[2/] Also, it was shown that the C'-fixing activity of the antibody produced by different individuals could vary considerably, particularly during the early phase of immunization.

In the experiment to be described, Wallace immunized three rabbits (Nos. 88, 89, and 138) with bovine serum albumin. Serum was obtained from these animals and quantitative precipitin tests performed to determine the antibody content in terms of antibody nitrogen. Quantitative CF tests were done to determine the weight of antibody required to fix 50 out of 100 complement (C') units introduced into the fixation mixtures. The unit of C' used in this instance was the 50 percent unit, or the amount of C' required to lyse 50 percent of a standardized suspension of sensitized sheep red blood cells.

It will be seen in table 1 that for rabbits 88 and 89, approximately 2 micrograms of antibody nitrogen were required to fix 50 of the 100 C' units introduced into the system whereas almost 4 micrograms of antibody nitrogen produced by rabbit 138 was required to fix the same amount of complement. In practical interpretation, this finding demonstrated that it would be possible to obtain identical CF titers with a number of early-course serums, and make the false assumption that the antibody contents were the same. As shown here, it would be entirely possible to have some sera having almost twice as much antibody, in terms of actual weight units, and show identical C'-fixing titers.

The same rabbits were then given a second course of injections, animals were bled, and the assays were repeated. It will now be seen that rabbits 88 and 89 fixed 50 of the 100 C' units with only 1 microgram of antibody nitrogen and rabbit 138 approached the same level of C'-fixing efficiency. Again, thinking of these results in practical terms, it would be possible with an animal such as 138, to observe a threefold increase in titer and attribute this to an increase in antibody content. However, it is obvious that it would be possible to observe such an increase in titer without any actual increase in the amount of antibody having occurred. It would appear, therefore, that the antibody formed early in the course of immunization, i.e., acute phase antibody, has a lower C'-fixing capability on a weight basis than that occurring late in the course of immunization, i.e., convalescent antibody. In studies on the antibody response of an animal to an infectious agent by the CF test, there may be a question as to what is being measured when an increase in titer is obtained. Is it due to an increase in antibody content, or is it

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2/ Numbers in parentheses refer to Literature Cited at the end of this paper.

perhaps attributable to an increase in the C'-fixing ability of the antibodies being produced?

The reasons for the poor C -fixing quality of the antibody produced in rabbits early in the course of immunization are not known. This is unfortunate because, in the field of veterinary serology, it is often necessary to work with species of animals that apparently produce antibodies with poor C'-fixing characteristics. Cattle, chickens, horses, possibly swine, sheep, and goats appear to be peculiar with respect to the C' -fixing activity of the antibodies they produce. As a consequence, the use of the C'-fixation test has been of somewhat limited value in the field of veterinary medicine.

In cattle, the poor C'-fixing characteristic of antibody has been so marked that it has often been referred to as a "non-complement fixing" antibody producer. This is incorrect, because the CF test has been used diagnostically in a variety of different cattle disease situations. In most instances, however, the tests were done with antigens that were particulate in nature. It would seem that the physical state of the antigen may also some influence on the ability of bovine antibody to fix C'. Attempts to demonstrate bovine antibody to viral agents by the CF test have generally been most unsatisfactory. In order to get around this problem with bovine and chicken antibody, Rice of Canada (2) developed the indirect complement fixation test. This has proven satisfactory, but its use has been somewhat limited because of its complexity. Several investigators have reported that positive CF reactions could be obtained if the bovine serums under test were used without being heat-inactivated as is usually done. Recently Boulanger and Bannister (3) in Canada and Knight and Cowan (4) in Kenya reinvestigated this problem independently and found a satisfactory test could be obtained with certain bovine antibody systems if a small amount of normal bovine serum was included in the test reaction mixtures.

Because these initial studies were done on rather ill-defined antigen preparations, the author initiated studies in which a better characterized antigen, i.e., crystalline egg albumin, was used. Serums from steers immunized with this material were examined for precipitating and C'-fixing antibodies by quantitative techniques. In the routine type CF test, five 50 percent hemolytic units of C' were used, and little evidence of CF could be obtained unless normal bovine serum was included. However, when tested by quantitative CF test methods, fixation of a low order of magnitude could be demonstrated even without added normal bovine serum. Results of such an experiment are presented in figure 1. In this assay, fifty 50 percent hemolytic units of C' were added to a constant amount of antibody followed by varying amounts of antigen. In this instance, 0.86 micrograms of antibody nitrogen were used. Following a suitable incubation period, residual C' was determined and the number of C' units fixed was calculated. The lower curve represents the fixation of C' obtained without added normal bovine serum. The upper curve shows the fixation that occurred when a 1:100 dilution of normal bovine serum

was included. This experiment demonstrates that bovine antibody, even in the absence of normal bovine serum, does fix C' however, it does so very poorly unless normal bovine serum is added.

As a sidelight on CF reactions, it is of interest to note in figure 1 that less C' was fixed with the larger quantities of antigen. This is a rather typical CF pattern that is obtained with pure antigen preparations and clearly demonstrates that the fixation reaction may be inhibited by an excess of antigen. From a practical point of view, this observation stresses the advisability of performing block, or two-dimensional, CF tests for the purpose of determining the optimal antigen amount for routine testing purposes.

Returning to the problem of antibodies with poor C'-fixing characteristics and the enhancement by normal serum, very little is known about the phenomenon. Studies are in progress at several laboratories to elucidate the reaction. It may be anticipated that a clarification of this problem will greatly expand our diagnostic capabilities for a number of animal diseases and probably provide information of a fundamental nature on the mechanism of the CF reaction.

The various observations just discussed are some of the many illustrations that could be cited to point out the fact that antibodies are heterogeneous in their immunological reactivity. Examples of physical and chemical heterogeneity could also be presented if time permitted. It has been pointed out that antibody reactivity may change with time in an indivisual animal. It may also vary from individual to individual within a species, and also from species to species. In the interpretation of serological results, one should think not only in terms of quantitative differences that may occur but also how the qualitative characteristics of the antibody may influence serological results.

Up to this point the discussion has dealt with reactivities of antibodies to pure antigen preparations. However, infectious agents are rarely antigenically simple, but are usually composed of multiple antigenic components. In addition, antigen and antibodies may occur which are not attributable to any antigenic components of the infectious agent, but are apparently due to the effect of the agent on the host. This is perhaps best illustrated in the case of syphilis where the appearance of antibody to tissue constituents, which have no antigenic relationship to the infectious agent, has provided the diagnostically important Kahn and Wassermann tests.

In the CF test, it should be appreciated that the test merely demonstrates that an antigen-antibody reaction has occurred. When a mixture of several antigens and antibodies are tested, there is no indication which, or to what extent, different immunological components are influencing the results. As a consequence, when mixed antigen-antibody systems are examined, there is no guarantee that the results indicate a measurement of the things either intended or thought to be measured. Too often the possibility is ignored that

mixed systems are being worked with, and as a result, erroneous conclusions may be reached because of failure to establish precisely what is being measured.

In recent years some extremely effective tools have become available for resolving antigenic mixtures. In one test, antigen and antibody are diffused through agar gels toward one another, and when they meet in suitable proportions, a line or band of precipitate is formed. Where several antigens and antibodies are present in the reagents, multiple bands of precipitate will occur because of differences in diffusion rates and concentrations of the reactants. This procedure was initially developed by Oudin, Elek, and Ouchterlony. The resolving power has been further increased by Grabar, who introduced electrophoresis into the technique. Various modifications of agar gel precipitin tests have been used extensively to resolve complex antibody-antigen systems and to identify antigenic components by comparison with known systems. Recently they have been applied to various virus systems, and in most of the ones studies multiple antigenic components have been demonstrated.

An extremely complex situation that may occur with virus preparations is illustrated in figure 2 by the agar gel precipitin reactions occurring with African swine fever reagents. The antigen in the center well was prepared from the fluid phase of swine kidney cell cultures infected with African swine fever virus. Thes fluids were concentrated approximately eighty fold by dialysis against a high molecular weight polyethylene-glycol compound. Wells 1, 3, and 5 received serum from swine that survived an infection with a tissue culture modified strain of African swine fever virus. Two to four bands may be seen between the antigen and serum wells. In the original agar preparation, four bands were clearly evident with all three sera. Wells 2 and 4 contained hog cholera immune serum and well 6, serum from a normal pig. These sera gave no reaction with the antigen preparation.

Figure 3, shows results of testing the same sera except that the antigen in the center well was prepared by extracting the washed cell debris from infected cultures with dilute sodium hydroxide. Two bands of precipitate are clearly evident in the photograph, but two additional weak bands were seen in the original plate. It should be mentioned that control antigens prepared in the same manner from uninoculated cultures have never given any reaction with these antisera.

Thus, at least four antigens were found in the fluid phase of infected cultures, and four were extracted from the cell debris. Identity experiments, performed to determine whether the antigens in the two phases were the same or not, indicated that some were and some were not. From these experiments, it would appear that some five to seven different antigenic components and their corresponding antibodies occur in African swine fever, and this is a minimal estimate of the number of components. It is interesting that despite the many different antigens and antibodies demonstrated, it has not yet been

possible to demonstrate any neutralization of the virus with the serum of swine surviving the disease.

As might be expected, CF reactions were readily obtained with such antigen and antibody reagents, but obviously it was not possible to properly assess the significance of a positive reaction. What these antigens are, where they come from, or what influence they have on the infectious process are unanswered questions. To answer these, it will be necessary to fractionate these mixtures and obtain pure preparations of each. When this is done and proper immunological methods applied, it may be possible to assign a specific function to some of the antigens and their corresponding antibodies.

Because such complex situations may occur with various infectious agents, one of the major endeavors in immunochemistry has been directed toward the fractionation of such mixtures and the preparation of purified components by whatever physical or chemical means available. In order to obtain the full value of serological tests, it is essential to establish that we are measuring the component we wish to measure, and not some incidental component or contaminant.

As an example, mention is made of some work on rinderpest done in Kenya by White and Cowan (5). It was found that a specific precipitating antigen could be demonstrated in lymph nodes of cattle infected with rinderpest virus. This antigen could be demonstrated by performing agar gel precipitin tests or CF tests, and these tests proved to be extremely valuable diagnostic tools. However, there was a question of what was being measured and whether or not these reactions were attributable to intact virus particles, or perhaps to some other antigenic component. To investigate this, lymph nodes were collected from infected animals and the extract was ultracentrifuged. The supernatant fluid and resuspended sediment were examined for infectivity and precipitating activity. As shown in table 2, the infectivity was mainly in the sediment, which did not give a precipitin reaction, whereas the precipitating antigen was found in the supernatant fluid.

This finding clearly indicated that the infectious particle and the precipitating antigen were physically different but revealed nothing about the possible immunological relationship of the soluble precipitating antigen and the infectious virus particles. To investigate this, a lymph node extract was chromatographed on a DEAE-cellulose column and a relatively clean soluble antigen preparation obtained. An optimal amount of this material was used to absorb a rabbit hyperimmune serum, which was then tested for both neutralizing and precipitating activity. It was found that although all the precipitating antibody had been removed, there was no reduction in the neutralizing activity. Therefore, it was concluded that the precipitating antibody was not directed against a viral antigen, or at least an antigenic component that was involved in the neutralization reaction. It was evident that it was not possible to utilize the precipitin or the CF test as a measure of neutralizing antibody. Until a specific viral antigen is prepared, the neutralization

test is the only test available for demonstrating antibody that is clearly directed against virus particles.

This discussion may have given an impression that the CF test is a poor procedure for measuring antibodies or antigens, but that is quite contrary to the impression one should carry away. All serological procedures have their limitations, and the CF test is probably no worse than any of the others. This applies to neutralization tests as well. Too often it is accepted that if an animal is immunized with a preparation that contains virus, the contaminating materials will not influence the neutralization test results as they will a CF test. However, one should consider some of the work by Rubin (6) on Rous Sarcoma virus, in which he found that the virus apparently could be neutralized with antiserum to normal tissue components. The neutralization observed was not due to antibody against the virus, but apparently to antibody directed against the virus receptor cells or sites. So even in performing the neutralization test, one should clearly establish what is being measured.

It is evident that there are a great number of possibilities for determining virus structure by immunological procedures, and much progress may be anticipated along these lines. With respect to antigenic materials like those demonstrated in African swine fever and in rinderpest, it is difficult to establish the source of these materials. Are they virus subunits, degenerated or denatured viral antigens, virus precursors, or what are they? This is unknown, but some may prove to be autoantigens, or altered cell materials produced by the infected host cells as a result of the infectious agent.

One might recall the work cited by Stormont on the production of toxin by diptheria organisms. In this instance, the virus infection alters the metabolic or synthetic pathways of the bacteria to produce the toxin that is not produced by the normal bacteria. Perhaps there are reactions like this occurring in the animal body which one might think of in terms of synthetic misfires, where foreign materials may be produced by infected cells as a by-product of the virus infection. Regardless, it is clear that until the nature of these components is understood, there will certainly be a void in our knowledge of the host response to infectious agents.

As we delve deeper into the minutiae of agent structure and host response, sharper serological tools will have to be applied to demonstrate the small differences that may be so necessary to understanding. The precise methods of immunochemistry provide such tools, and it may be anticipated that they will become of increasing value and, in fact, essential in resolving many of the problems pertaining to host-agent relationships.

TABLE 1.--Fixation of C' by first-course and second-course rabbit-antibovine serum albumin antibody. [1/]

Rabbit No.	μg. (microgram) of Ab N fixing 50 out of 100 $C'H_{50}$
First course of immunization:	
88	2.21
89	2.11
138	3.96
Second course of immunization:	
88	1.15
89	1.00
138	1.28

[1/] See the work of Wallace and others (1).

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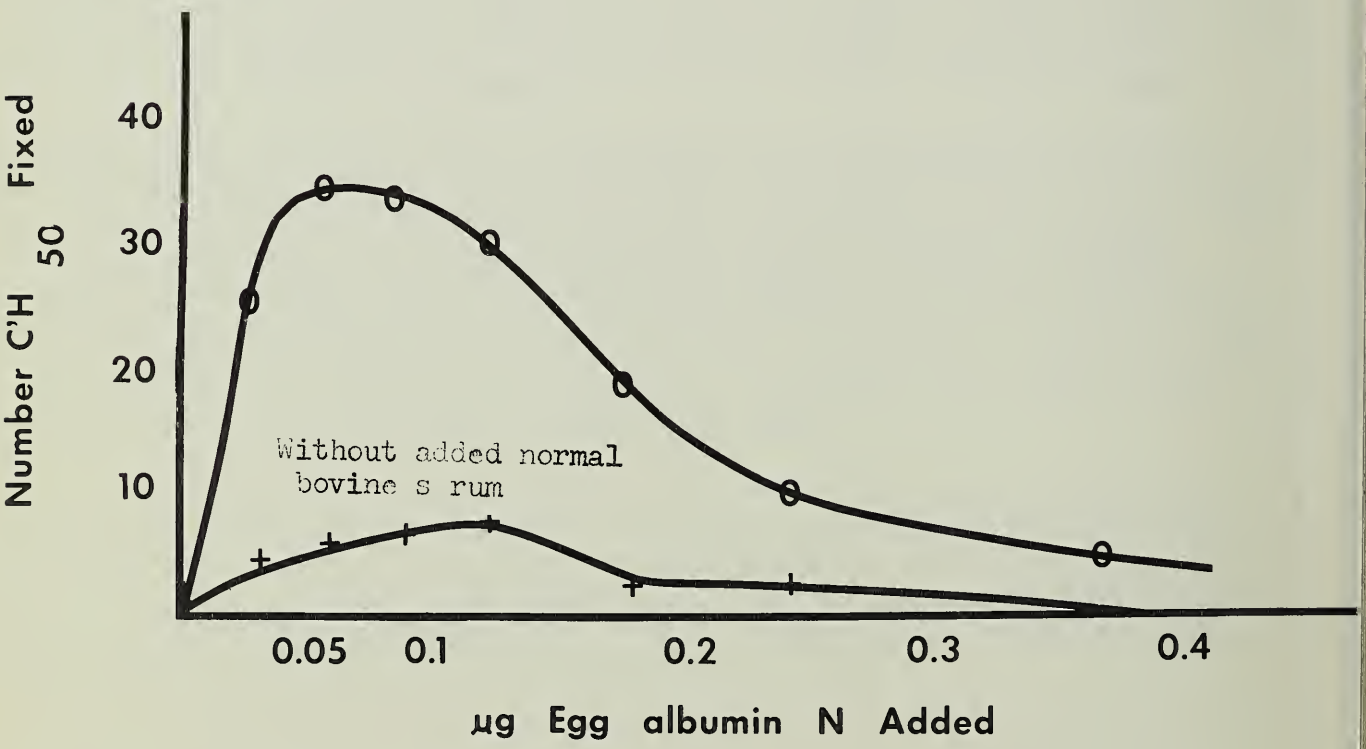

Figure 1.--Vixation of C' by varying amounts of egg albumin and 0.86 µg. of bovine anti-egg-albumin antibody nitrogen.

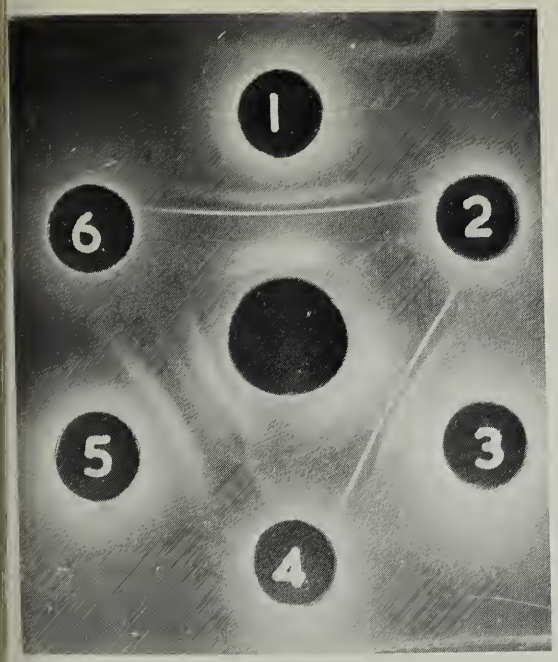

Figure 2.--Agar gel precipitin reaction between antigen prepared from the fluid phase of tissue cultures infected with African swine fever virus and various swine serums. Wells 1, 3, and 5 contained serum from swine surviving an infection with modified African swine fever virus. Wells 2 and 4 received swine anti-hog-cholera serums. Well 6 received normal swine serum. Center well contained the antigen. A mold contaminant is around well 3.

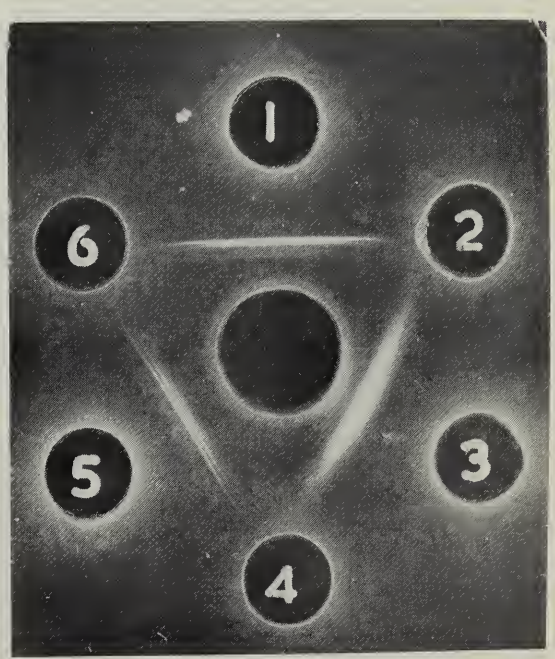

Figure 3.--Agar gel precipitin reaction between antigen extracted with dilute sodium hydroxide from the cell debris of tissue cultures infected with African swine fever virus and various swine serums. Wells 1, 3, and 5 contained serum from swine surviving an infection with modified African swine fever virus. Wells 2 and 4 received swine anti-hog-cholera serums. Well 6 received normal swine serum. Center well contained the antigen.

HOST REACTION TO DISEASE: APPLICATION OF RADIONUCLIDES

Leo K. Bustad, D. V. M., and R. O. McClellan, D. V. M.[1/]

Almost 4 decades ago, the classical experiments of George de Hevesy (1923)[2/] introduced the use of radioisotopes in biological research. This Nobel prize winner studied the absorption and translocation of an isotope of lead in a bean plant. Hevesy and Hofer (1934) determined the total water content and half-life of water in their own bodies using deuterium (heavy hydrogen), and a year later, Chiewitz and Hevesy (1935) studied the distribution and excretion of radioactive phosphorus in the rat. Since these pioneering works of George de Hevesy and his coworkers using isotopes as "indicators" in physiological studies, many ingenious applications of the tracer principle have taken place and, in fact, have revolutionized the biomedical sciences.

Hevesy and a relatively few workers have been joined since the 1940's by a host of scientists and technicians representing many disciplines, including physicists, chemists, biologists, and also clinicians in dental, medical, and veterinary fields. The result has been a refreshing cross-fertilization with different approaches to problems beyond the reach of any one of these disciplines.

Because of the interdisciplinary approach in the application of radioisotopes, it has become possible to determine in the normal and in the diseased individual, not only the quantity and distribution of elements in the body and areas of location, but also the rates at which certain functions are proceeding, and the chemical pathways involved. The great progress made during the past decades may be only a prelude to great advances in tomorrow's biomedical research advances, for which the only limit is the ingenuity of the scientist and the technologist.

1/ Biology Laboratory, Hanford Laboratories, General Electric Company, Richland, Wash. The work reported here was performed under Contract No. AT (45-1)-1350 between the Atomic Energy Commission and the General Electric Company.

2/ The oral presentation was accompanied by 11 additional photographs that are not included in this publication. The year in parentheses after author's name is the key to the reference in Literature Cited at the end of this paper.

We will discuss something about radionuclides and how they may be used. First, however, let us review what we have learned from physical scientists.

Until this century, each element in nature was believed to occupy an unchallenged place in the periodic table. It was not until just a few decades ago that it was learned that certain elements possessed different masses but shared the same atomic number and had the same chemical behavior. The term "element," then, is more correctly a collective expression referring to a family of atoms.

The difference in mass observed among the isotopes of a given element is due to a loss or gain of neutrons. Today more than 1,100 natural and man-made isotopes or more precisely, nuclides according to Glasstone (1958), have been identified. The nuclei of some of these isotopes are stable, while others disintegrate or decay spontaneously in the course of time to form different nuclei. The phenomenon of radioactivity is associated with a disintegrating isotope and with the emission of an alpha or beta particle and often a gamma proton. Each radioactive isotope has a characteristic half-life, particle emission, and energy spectrum. The isotopes of hydrogen show the greatest difference in mass. Deuterium, a stable isotope, has twice the mass of ordinary hydrogen; tritium, which is radioactive, has three times the mass of protium (fig. 1).

Fortunately the radioactive isotopes generally resemble the stable element in chemical behavior. They will, however, have different rates for change of state, mobility, and chemical bonding. These differences are proportional to the ratio of their isotopic weights and will, therefore, be greatest with the isotopes of hydrogen and generally negligible with elements of higher mass. These different rates and mobilities of the elements of lower mass, however, will not seriously affect our tracer studies in applied medical research.

DETECTION METHODS

A large variety of instruments have been and are being developed for radiation detection. Measurement of the radiation emitted by a radioisotope depends on the excitation or ionization that occurs as the radiation (alpha and beta particle or gamma ray) traverses matter. The three principal methods of detecting radiation depend on:

1. A chemical change in matter--for example, using photographic emulsions with I^{125} localization in the thyroid and Sr^{90} in the bone.

2. Collection of ions produced in a gas by traversing particles in an electrical field--for example, gas ionization chamber or Geiger-Mueller (G-M) tube. Figure 2 shows a G-M tube assembly in position on a sheep for external monitoring of I^{131} in the thyroid.

3. Production of luminous scintillations in optically clear solids or liquids by primary or secondary charged particles. The application of this method is seen in figure 3, which shows a two-probed scintillation detector and accompanying amplifiers, discriminators, and recorders for monitoring swine. A variety of amplifying, counting, and recording equipment are utilized with gas ionization chambers and scintillation detectors.

One of the most important recent advances in practical instrumentation is the development of the gamma spectrometer. This device is used to determine the pulse height energy spectrum of radioelements emitting penetrating radiation. It allows the detection of one gamma-emitting element in the presence of a number of others. One of the instruments we use for these measurements in Hanford Laboratories is illustrated in figure 4, which shows a steel tube equipped with a 9-inch by 4-inch NaI crystal in an underground monitoring facility.

These principal methods of detection and measurement are applicable in locating and determining the distribution of a radioactive element, either in a molecule or subcellular structure, a cell, a tissue, an organ, or even the whole body.

An interesting and useful detection method, using a combination of instruments and methods, is called scanning. Scanning is defined as detecting and recording the localization, distribution, and concentration of radioisotopes in the living body from some point outside the body. The early method of point-to-point scanning with a crude hand-held detector of low sensitivity has now been replaced by scanners. These instruments can automatically scan body areas or the whole body with reasonable efficiency and record the data mechanically, electrically, or photographically - even in color. Such an instrument was recently described by Mallard and coworkers (1961), in which radioactive arsenic, a positron emitter was used for locating brain tumors. The scanning results were plotted on a strip chart recorder.

For the sake of completeness, we must mention another interesting detection method--neutron activation analysis. It is both a quantitative and qualitative method of measurement, in which elements are bombarded by a known flux of neutrons, thereby becoming radioactive. The disintegrating isotopes produced can be characterized by analyzing their energies and half-lives. By this method, one can quantitate with great accuracy the original amount of the certain elements present if one selects the appropriate time and flux for neutron exposure and makes proper analysis.

These various detection methods will become more meaningful as we discuss them in relation to various biomedical applications.

APPLICATION OF INTERNAL EMITTERS FOR STUDYING PHYSIOLOGICAL RESPONSES OF ANIMALS OR ORGAN SYSTEMS

Animal response to disease may be manifested by general or localized reaction. Some physiological measurements that may be applicable in determining a general or overall response of an organ or system to disease are:

1. Determination of "Lean Body Mass" and Total Body Potassium.

As a reference on which to base many physiological measurements, "lean body mass," described by Behnke (1942), rather than body weight is a useful concept and criterion. Some evidence has appeared suggesting that the normal K^{40} of the body as determined by whole-body monitoring is a reasonable indicator for the "lean body mass" (and, of course, for total body potassium). Body potassium levels may prove to be useful in a number of clinical studies and can be determined in animals by using the apparatus shown in figure 4.

2. Measurement of Body Components, Including Total Body Water, Total Exchangeable Sodium and Potassium, and Extracellular Water.

All these measurements are based on the principal of isotope dilution; that is, the extent to which a radionuclide is diluted in a solvent constitutes the measure of the volume of the solvent. The radionuclide used and results for the foregoing parameters are listed in table 1. Exchangeable sodium and potassium can also be determined by utilizing isotopes of these elements. As more knowledge is gained on changes in body composition and their convenient detection, a broader approach to the understanding of disease will be possible.

3. Metabolic Rate.

The various thyroid function tests are being used as indicators of metabolic rate and include percentage of administered I^{131} concentrated in the thyroid, rate of uptake in and release of I^{131} by the thyroid, amount and rate of I^{131} excreted in the urine, and amount of PBI^{131} in the blood. If properly interpreted, tests employing radioiodine are generally considered superior to the classical basic-metabolic-rate test for the detection of hyperthyroidism and a number of other thyroid disorders.

The thyroid, of course, reflects a change in a number of diseases or abnormal states. A whole-body monitor, employing a shield over the thyroid may also be utilized. Utilizing such a procedure, we have determined the body and thyroid burden and its change with time in sheep. The methods we have employed for simply determining thyroid uptake in sheep and swine are illustrated in figures 5 and 6.

4. Lung Function.

Some pulmonary function tests lend themselves quite readily to *in vivo* radionuclide studies. A recent report by Ball and others (1961), showed that Xe^{133}, a gamma-emitting and readily available inert gas, was suitable for studies of respiration. Simultaneous count rates over selected areas of the chest were recorded on a multiple channel tape recorder while the patient inhaled a known concentration of radioactive gas from the spirometer. The tapes were rerun through ratemeters and the data recorded on charts to provide functional data on regional areas of the lung. Xe^{133} can also be injected intravenously, and its appearance in the lung can be observed for determining perfusion in various regions of the lung. (Radiokrypton has also been used for pulmonary function tests.)

5. Liver and Gastrointestinal Function.

Abnormal function involving disturbances in liver function or in intestinal absorption of fats and proteins may be readily detected by employing radionuclide techniques. Taplin and coworkers (1955) introduced the radioactive rose bengal liver function test, which has been widely accepted. The dye is excreted almost entirely by the polygonal cells of the liver. The test has been simplified since it was introduced so that following I.V. injection of the I^{131} rose bengal, frequent counts are made over a vascular area of the head for 20 to 30 minutes and the disappearance curve determined. In subsequent work, the I^{131} rose bengal test for sheep was modified. A sample plotting of our data obtained from sheep through *in vivo* monitoring of a vascular area and by frequent blood sampling is shown in figure 7. The monitoring equipment is shown in figure 8. The *in vivo* detection shows an apparent slower disappearance of I^{131} because of the accumulation of the I^{131} label in the tissues being monitored and consequent increase of background.

Scanning techniques can also be utilized with this procedure to determine abnormal gall bladder function or liver pickup and release.

Detection of loss of protein from the gastrointestinal tract, a major factor in hypoproteinemia, has only recently been satisfactorily resolved using a labeled normal metabolite. Blood albumin labeled with Cr^{51} is injected intravenously and the excretion pattern noted. In patients with gastrointestinal disease, Waldmann (1961) observed that 4 to 21 percent of the dose appeared in the 4-day stool and none in the urine. Less than 1 percent appeared in the stool of the controls during the same period.

A number of malabsorption syndromes can be detected using an assortment of labeled metabolites. Simultaneous oral administration of I^{131}-labeled oleic acid and I^{131}-glyceroltrioleate (triolein) have been successfully applied clinically. In pancreatic disease oleic acid absorption is normal, but triolein absorption is impaired. In other conditions, for example sprue, absorption of both of these substances is impaired.

Gastric emptying rates in the dog have been determined using Ir^{192}-labeled colloidal resin with external scanning. The procedure utilizes lead shielding and careful fluoroscopic positioning.

Of possible interest to some of you is a procedure for labeling food with poorly absorbed isotopes to determine the amount of food eaten by measuring the radioactivity of the feces. Thompson and Palmer (1960) in Hanford Laboratories have successfully used cobalt oxide for such determinations in rats.

6. Pancreatic Function.

Effective scintiscanning of the pancreas has only recently been reported by Blau and Bender (1961), who utilized the selenium analog of methionine Se^{75} as a substitute for sulfur. This compound was found to have a pancreas specificity equal to that of the natural amino acid. One hour after administration, up to 7 percent of the selenomethionine was found in the dog pancreas with concentrations eight or nine times that in the liver. The method may be very useful as a function test.

7. Splenic Scanning.

Practical splenic scintillation scanning, too, has only recently been developed, according to Johnson and others (1961). It is based on the selective trapping ability of the spleen for radioactive red blood cells. Since the spleen has a more efficient filter than the liver, a differential concentration is realized. Normal red cells may be modified either by coating (sensitizing) or by heating the cells at 49.8° C. for 15 minutes. The heated cell technique requires only 30 minutes for preparation, including the addition of Cr^{51}. Scintillation probes are used to monitor the radiation over the spleen, liver, and thigh, with the latter value being subtracted from the other two values to give net count. Conventional print-out on carbon-backed paper or photoscan technique may be utilized. Most of the radioactive cells are cleared within 4 hours but are detectable within the first 15 minutes. This technique demonstrates the size, position, and configuration of a spleen and it may identify accessory splenic tissue and serve as an index of common function of the liver and spleen.

8. Renal and Cardiovascular Function.

An extensive amount of work has been done with radionuclides in the study of the renal system and the cardiovascular system. One of the more recent renal function tests as reported by Winter (1956), employs I^{131}- labeled diodrast. Two lead-shielded scintillation counters placed over the kidney areas record the rate of accumulation and disappearance of the radioactive diodrast.

The various methods used in cardiovascular studies will be alluded to only briefly, although there has been an extensive application of isotopes in cardiovascular studies and in hematology. The reader is referred to Lajtha's (1961) book on this subject and to a number of other fine texts in nuclear medicine or isotopes.

Two methods used in cardiovascular diagnosis are:

1. Determination of blood volume by isotopic dilution techniques previously described.

2. Determination of circulation time from a vein to the peeriphery of a limb or from the periphery to the heart. Na^{24} or radioiodinated human serum albumin (RISA or I^{131} HSA) is often utilized for these tests. One such technique was utilized by MacIntyre and others (1952).

For determining cardiac output, several methods use I^{131} HSA as a tracer. Since this material is retained for some time in the plasma, it is possible to obtain output and blood volume simultaneously. Two methods that have been used clinically are:

1. Scintillation detection over the precordium and a continuous record obtained as the I^{131} HSA passes through the heart and into the circulation.

2. The concentration of I^{131} HSA in arterial blood samples is counted individually or continuously as the arterial blood flows through the counter. The dilution curve may be continuously recorded by a counting rate computer in conjunction with a scaler and recorder.

Cytological and Ion Movement and Transport Studies Utilizing Isotopes

One of the most graphic cellular studies that uses radionuclides is the demonstration of the manner and rate of cell proliferation in the intestines. Either P^{32}-labeled DNA (deoxyribonucleic acid) or H^3 (as tritiated thymidine)

Biochemical Studies With Isotopes Involving Product-Precursor Relationships and Metabolic Pathways

Isotopically labeled substances have revolutionized studies in intermediary metabolism. There are now thousands of chemicals available as shelf items and there are probably few, if any, organic compounds that cannot be isotopically labeled. Studies on metabolism in the rumen have received a great deal of much needed attention during the past decade. For references on this subject, the reader will find the publications of Annison and Lewis (1959), Kleiber (1961), and others of great interest and assistance. A good example of studies that can be performed is that by Block and coworkers (1951), in which S-labeled sodium sulfate added to the rumen is rapidly incorporated into protein, and the labeled sulfur appears in the milk protein within 3 hours. These workers suggested that the metabolism of sulfur compounds in ruminants may be influenced by the concentration of free sulfide as an active intermediate in the rumen. There is reason for optimism that tracer methods will provide the means for resolving many of the disturbances of rumen function that have plagued us for so many years in animal medicine.

Another important area of investigation is the use of C^{14} in labeled glucose to determine the alternate pathways in glucose metabolism. At least four pathways for glucose-6-phosphate metabolism are described. These include direct oxidation, glycolysis, and dephosphorylation to form glucose or conversion to glycogen. In comparing the results of a quantitative evaluation of the alternate pathways in the liver of a diabetic animal with a control, Ashmore and coworkers (1957) noted that insulin deficiency caused more of the glucose phosphorylate to be reconverted to glucose, less of it to glycogen, more of it oxidized to Co_2, and little change in the fraction that enters the glycolytic cycles.

Immunological Studies Applying Isotopes

A great variety of studies employing radioisotopes has been reported in the field of immunology. General methods have been developed for the labeling of active sites of antibodies using I^{131} and for following antibody synthesis after the injection of protein and cellular antigens, including I^{131}-labeled human serum albumin. An interesting application applying four, not one, isotopes was recently reported by Day and others (1961). The quadratic label employed I^{125}, I^{130}, I^{131} and I^{133} for controlled determination of localized antibodies. An example of the results of these workers obtained using a triadic label is shown in table 2.

The immune mechanisms can also be compromised by exposing the total body to external penetrating irradiation. This technique has been used in studying the immune mechanism and also as one step in a possible radiation therapeutic regimen in treatment of cancers, or the preparation of the host for a foreign graft or homotransplant, or both. Winchell (1961), working in

is useful for such studies. The advantage of tritiated thymidine is that it permits the identification of individual labeled cells. From such tracer studies it has been observed that cell birth occurs only in the crypts, and cell loss occurs only on the tips of the villi. When an animal is sacrificed within a few hours after labeling, only labeled cells are seen in the crypts and a definition of the time course of renewal in various segments of the intestines is possible, according to Hughes and others (1958).

Another interesting application of the tritium label was that of Baserga and Kisieleski (1961) who studied cell proliferation in tumor-bearing mice. They utilized tritiated thymidine injections in mice previously injected with suspensions of tumor cells in order to study the basis for the increased weight noted in some organs not directly involved in the neoplastic process.

Interesting information has also been obtained on a variety of cell systems. Cells of the hematopoietic cells have perhaps received the greatest attention ever since 1940, when Hahn and Hevesy injected P^{32} *in vivo* labeled red cells from one rabbit into another.

Cr^{51}, which possesses a convenient half-life, a slow elution rate, and is not reutilized, is a useful red-cell label, and is now used in chromium labeling as the method of choice for life-span studies. Another promising *in vivo* label is P^{32} diisopropyl fluorophosphonate (DFP). This substance combines irreversibly with cholinesterase in the red cells and is not re-utilized. It has also been employed for labeling white cells and platelets and is a most attractive label for determination of absolute life span. These labels are well described by Lajtha (1961).

In excitable tissue, i.e., muscles and nerves, the potassium concentration inside the cells is 20 to 70 times the external medium; chloride is up to 50 times more concentrated outside than inside, and sodium is 3 to 15 times more concentrated outside than inside. These ions and their movement by active and passive transport are responsible in large measure for the resting and action potential. Radioactive isotopes of these important elements have proved very valuable in ionic movement experiments, for they give direct information about ionic fluxes. It has been observed that there is a continuous net leakage of K^{42} from the nerve and that with stimulation, K^{42} efflux was increased 10 times. The amount lost about equaled the sodium influx. Na^{24} was also observed to cross plasma membranes in the resting fiber, and with nervous activity the influx was increased about 15 times, according to Keynes (1949).

the Lawrence Radiation Laboratory at the University of California, developed a method in which he attempted the selective irradiation of the lymphoid tissue using intravenous infusion of Y^{90}-chelated with DTPA (diethylene-triamine-pentaacetic acid).

Radioisotope Labeling Of Pathogenic Organisms and Insect Vectors

The successful labeling of bacteria, such as the localization of the tritium label in micro-organisms described by Painter and coworkers (1958) and the preparation of purified radioactive poliovirus particles by Hoyer and co-workers (1959), has given the microbiologist another valuable research tool. Benacerraf and associates (1959) applied the technique using P^{32}-labeled Escherichia coli and Staphlycoccus in order to determine the kinetics of blood clearance of these micro-organisms by the reticuloendothelial system. These workers noted that the liver concentrated the more readily phagocytized bacteria, while the spleen was more efficient in removing the less easily phagocytized bacteria, and that the rate of removal was dependent on the antibody level in the serum.

Van Tubergen (1961) used a combination of electron microscopy and radio-autography for examining tritium-labeled bacteria. The reader is referred to the many papers that are now available on this general subject of labeled micro-organisms and also a recent monograph by Sternberg (1958) on tuberculosis.

Isotopic labeling of metabolites provides a valuable means of studying the metabolic requirements of parasitic helminths and the interrelationships of the parasite and the metabolism of the host. Stoner and Hankes (1960) have successfully labeled trichinella and other parasites with tritium and also C^{14}. The application of this technique will contribute much needed information on the metabolism of trichinella and other parasites and that of the altered host, which may lead to development of antimetabolites or other control measures. The opportunities for productive research in this area appear almost limitless. Insects may also be labeled to determine their migratory range. In such a study at the University of California at Davis, the source of a fly nuisance was determined. Strings dipped in P^{32}-labeled sugar were exposed to a steer shed, and fly-collecting equipment was placed in various areas. Migratory patterns were in part determined, as was the "turnover rate" of labeled flies at the steer shed according to Smith (1961). For a summary of some entomological uses of radioisotopes, the reader is referred to the work of Lindquist (1958).

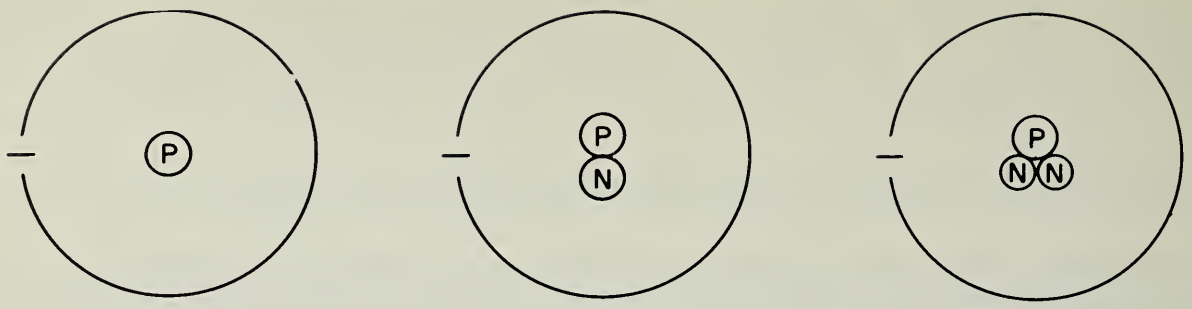

Figure 1.-- Diagrammatic structure of isotopes of hydrogen. Only tritium is radioactive.

Figure 2.--Thyroid monitor equipped with three Geiger-Mueller tubes for external monitoring of I^{131} in sheep.

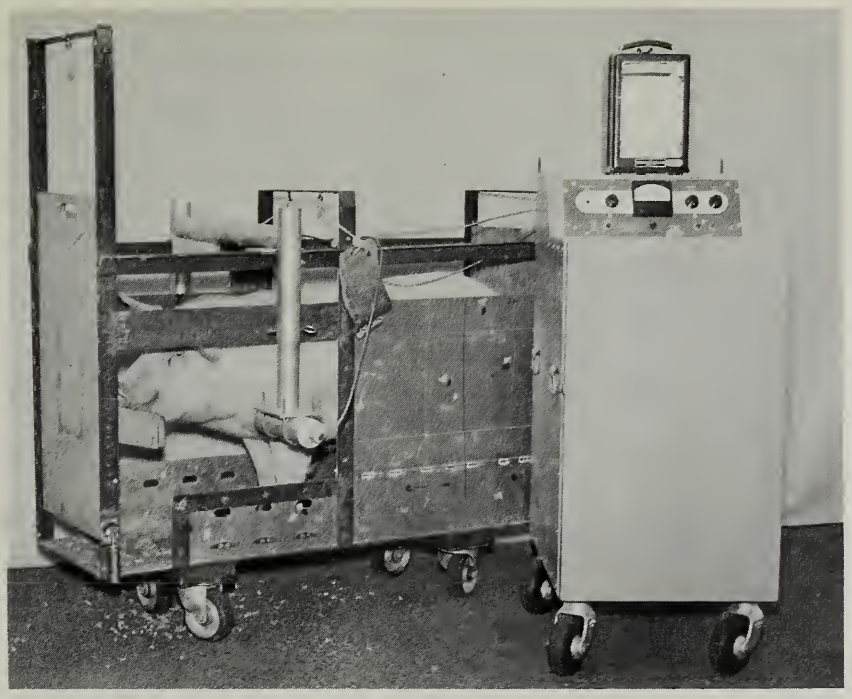

Figure 3.--Two-probed scintillation detector for external monitoring of I^{131} in the thyroid of swine.

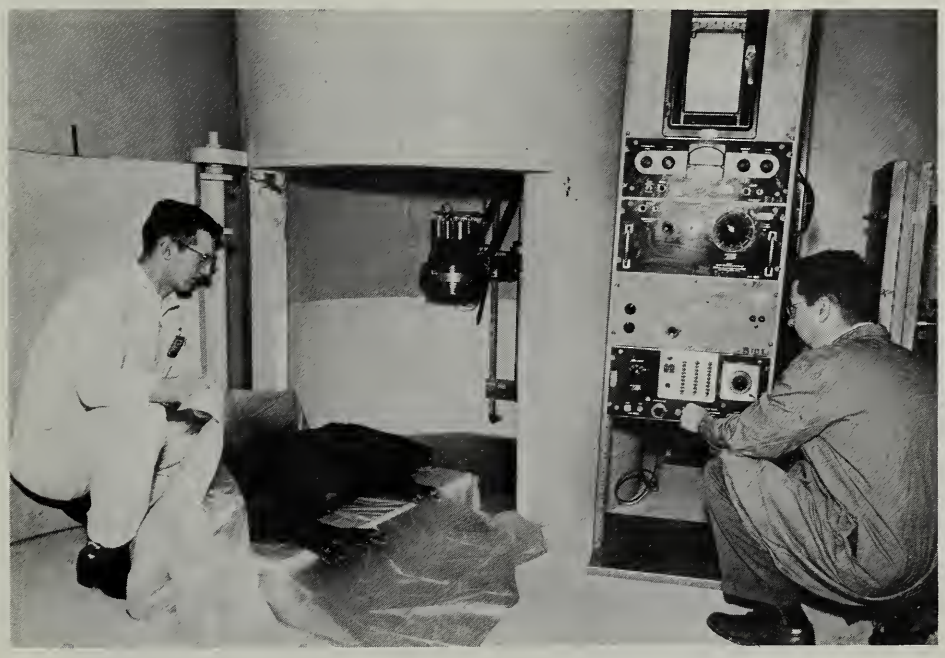

Figure 4.--Underground whole body monitor showing low background steel tube and a gamma spectrometer. An anesthetized miniature swine is about to be placed in the monitoring area.

Figure 5.--Sheep, with thyroid covered by a half-inch lead shield, is about to be placed in a whole body monitor.

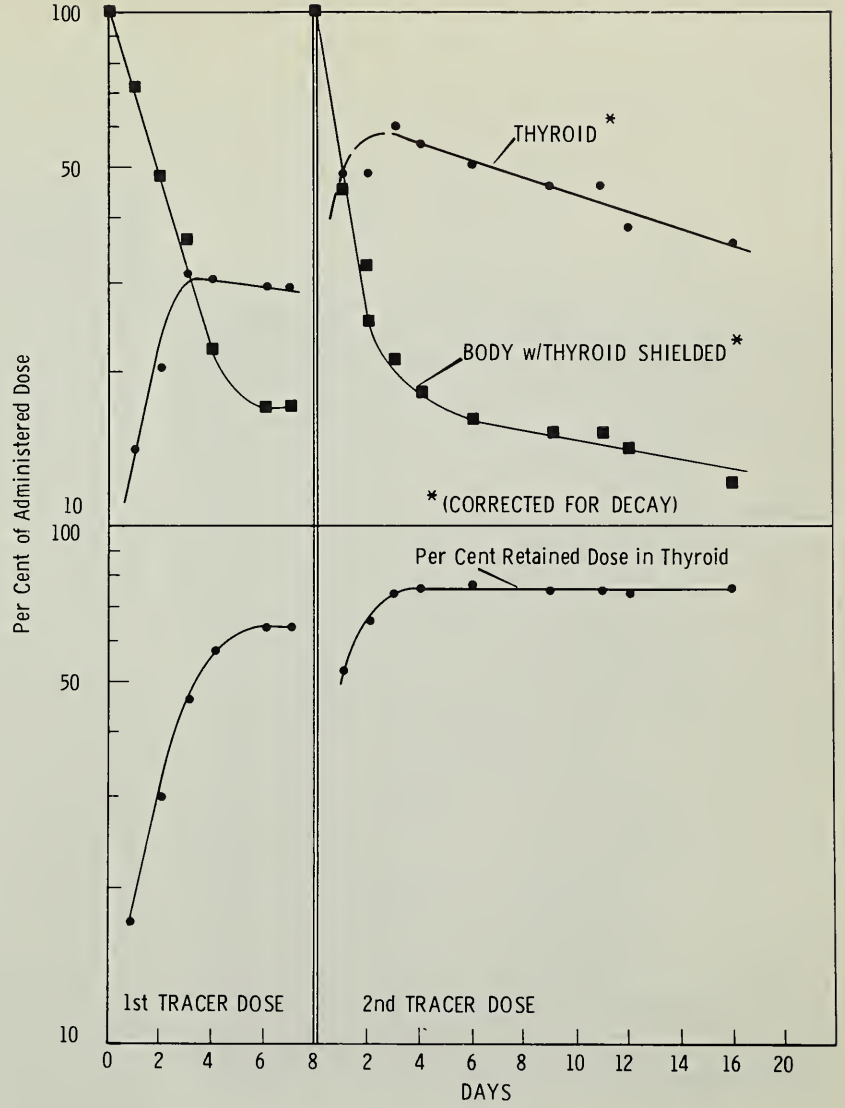

Figure 6.--Thyroid and body burden of I^{131} in newborn lamb measured by use of external monitoring with thyroid covered by lead shield. Shielded and unshielded counts are shown.

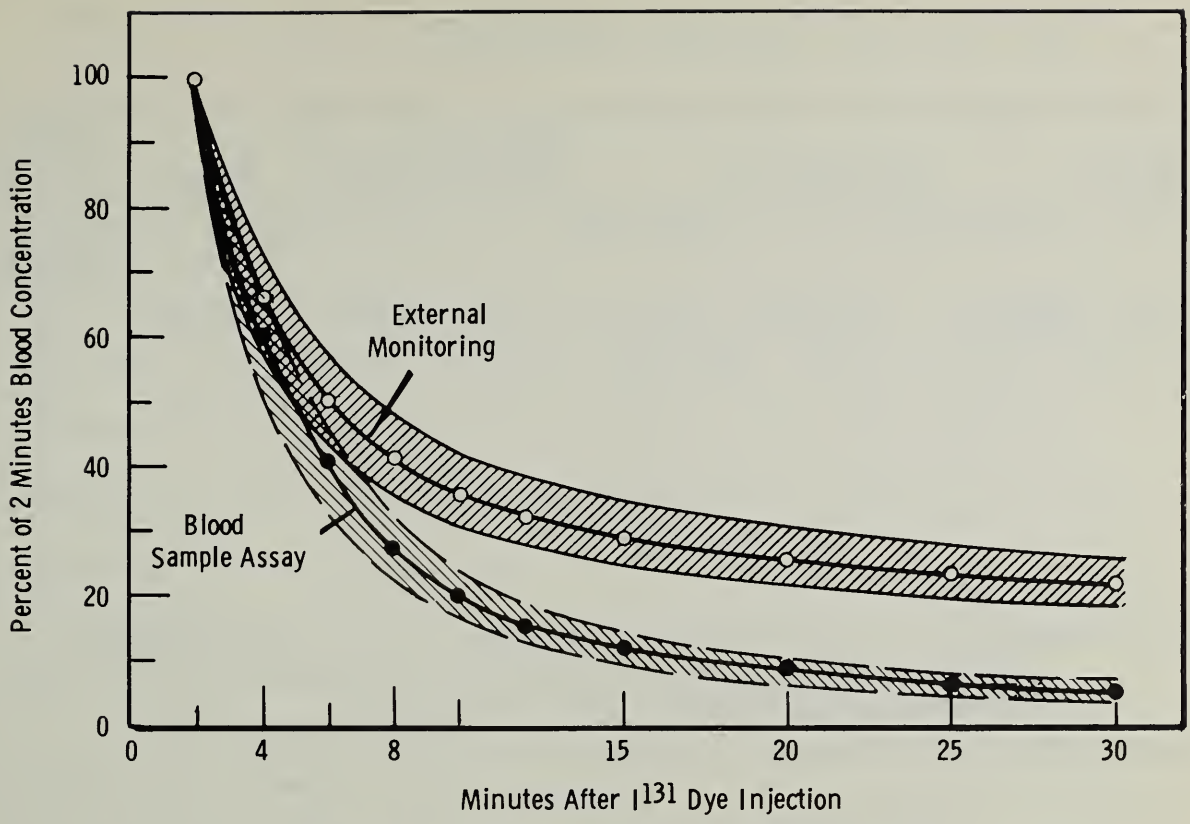

Figure 7.--Comparison of two methods for determining I^{131}-labeled rose bengal dye clearance in 14 normal sheep.

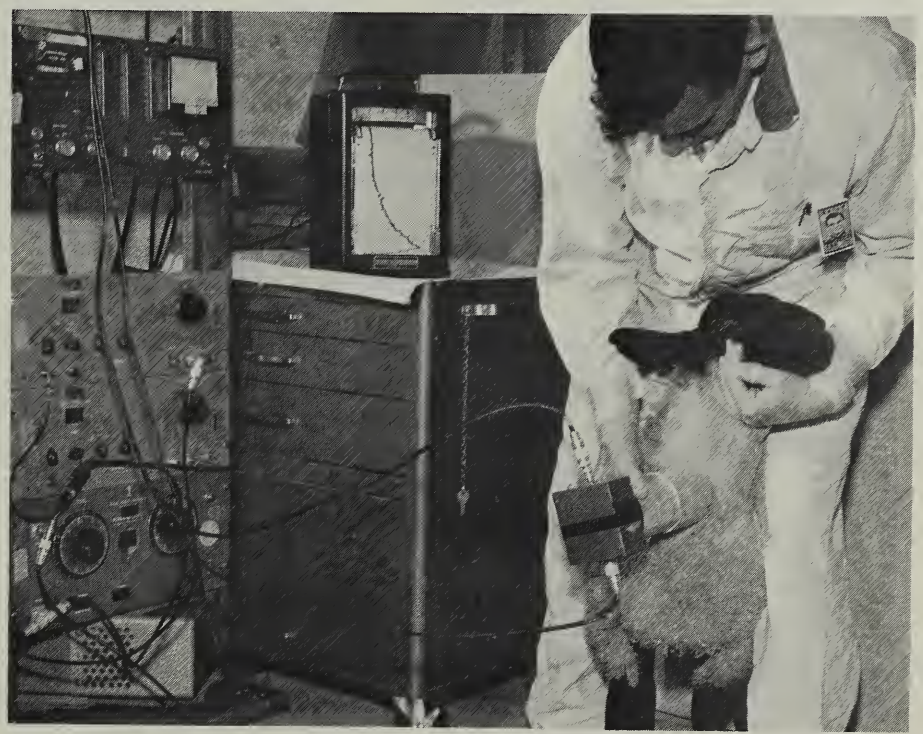

Figure 8.--Monitoring a vascular area in sheep for I^{131}-labeled rose bengal dye clearance.

Table 1.--Radionuclides methodology for determining extracellular water and total body water

Body constituent	Radionuclide	Route of Administration	Sample	Value (for man)
				Percent
Total body water--	Tritium	I.V. or oral	Plasma	60
Extracellular water--	Cl^{38}, Br^{82}, $S^{35}O_4$	I.V.	____	18

Table 2.--Comparative localization of antitumor (I^{131}) and antifibrin (I^{130}) antibodies above background localization (I^{133}) in different tumors within the same rat 1/

	Injected isotopic dose					
	Total dose per gram			Net dose per gram 2/		
Tissue		131	130	133	131	130
		Percent	Percent	Percent	Percent	Percent
Carcinoma of intestine	13.6	0.21	0.22	0.20	0.008	0.014
Hepatoma	11.4	.21	.12	.10	.10	.021
Liver	21.5	.26	.059	.044	.21	.015
Kidneys	4.4	.22	.019	.018	.20	.001
Spleen	1.1	.42	.098	.062	.35	.036

1/ From Day and others (1961).

2/ Total percent dose per gram minus percent dose per gram of I^{133} globulin.

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SECTION IV - HOST REACTIONS TO INFECTION

Lawrence O. Mott, D. V. M., discipline leader in virology, National Animal Disease Laboratory, presiding. 1/

SOME METHODS FOR THE STUDY OF MORPHOLOGICAL CELLULAR CHANGES CAUSED BY VIRUSES

Herman R. Seibold, D. V. M. 2/

A review of morphological cellular changes caused by viruses would be useful but not appropriate at this time. Much could be said about the renaissance in morphological research that has been developing in the past few decades. Biochemists, geneticists, microbiologists, and immunologists have become increasingly interested in the application of morphological techniques to relate their observations to the cell, the unit that holds the secrets of health and disease. We are marking the beginning of an expanded program of research in domestic animal diseases. Ideas devoted to progress are timely. One of those ideas is the role of method in the study of morphological cellular changes caused by viruses.

Let us pretend that the photographs of individuals in figure 1 are subjects for morphological study. Several facts are obvious. One individual is a child and the other a woman. Some details of the environment also are shown. However, many important facts concerning these individuals cannot be established by study of the pictures. It cannot be determined if the child is a

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1/ The following paper was also presented at the symposium but was not available for publication: Modification of the Host to Injury by Host-Parasite Factors, by Dennis W. Watson, Ph. D., Medical School, University of Minnesota, Minneapolis, Minn.

2/ Plum Island Animal Disease Laboratory, Animal Disease and Parasite Research Division, Agricultural Research Service, U.S. Department of Agriculture, Greenport, Long Island, N. Y. The author is now head of the Department of Pathology and Parasitology, School of Veterinary Medicine, Auburn University, Auburn, Ala.

boy or girl. Familial relationship of the child to the woman is not shown. Since the original photographs were color transparencies, it is logical to infer that the two people are not the same individual. Color photography had not been developed when the woman was a child! A series of pictures would provide more information, especially if taken over a period of years and were action shots of the individuals engaged in their daily routines. Such a series might be revealing about a variety of things, including habits, personality, preferences, education, occupation, financial status, social position, philosophy, morals, and religion.

The simile drawn between the foregoing situation and the study of limited histologic or tissue culture specimens may be farfetched. Yet it is true that morphological research often is done with a few specimens selected from "significant" organs of "typically" affected animals at the "right" time. Such an approach ignores the kinetic aspects of pathological conditions--their inception, development, and termination. To illustrate advantages of the comprehensive method in pathological research, some observations made at the Plum Island Animal Disease Laboratory will be presented.

A study of rinderpest was being made to develop a working knowledge of this dangerous exotic disease of cattle. Infected cell cultures of calf kidneys and of infected cattle were available for morphological studies. Among other things it was decided to make microcinematographic films of the cytopathic effects of the virus in calf kidney cells. The necessary equipment was set up and preliminary visual observation was begun.

The first problem encountered was the recognition and identification of the earliest morphological effects of the virus. Groups of large stellate cells developed in the infected cultures. These cells were observed in the coverslip cultures prepared for microcinematography before definite cytopathic effect was recognized in prescription bottle cultures. It seemed logical to assume that the stellate cells represented early cytopathic effect. Would it not be more easily recognized with phase microscopy of coverslip cultures than with conventional low-power microscopy of unstained prescription bottle cultures? The question was resolved in the negative by daily comparison of infected and noninfected cultures made on small coverslips in Leighton tubes.

The cultures harvested at daily intervals were fixed, stained, and examined in detail. Islands of large stellate cells (fig. 2) developed to the same number and extent in both uninfected and infected cultures. This observation raised another question. Were the large stellate cells a different type than the smaller compactly arranged cells composing the bulk of the culture, or were they the same cell just spread out in areas of less dense cell population? This question was resolved by making a microcinematographic film of a developing uninfected culture. The large stellate cells were present in scattered distribution from the beginning and underwent mitosis much less frequently than the smaller cells. The latter grew compactly in radial fashion from small original cell clusters and finally herded the large stellate cells into islands illustrated in figure 2. Thus, it was concluded

that the large stellate cells were a different type than the smaller ones.

Later microcinematographic studies on calf kidney cultures infected with foot-and-mouth disease virus provided evidence that the large stellate cells are histiocytes. Under conditions of severe culture destruction, these cells moved sluggishly and phagocytized cellular debris. They also appeared to be susceptible to the virus but to a lesser degree than the smaller cells.

When these questions were settled, the preliminary observations on the cytopathic effect of rinderpest virus were continued. It is known that tissue culture infected with rinderpest virus produces cell syncytia, i.e., large cell masses with multiple nuclei and a common cytoplasm (1).3/ A cell with four nuclei was seen in a coverslip culture harvested 5 days after infection. However, cells with two and three nuclei were easily found in the control cultures. Not until the sixth day after infection were significant morphological alterations seen. There were a few syncytia large enough to be distinguished from binuclear and trinuclear cells normally present and some cellular debris from the necrosis of individual cells. By the eighth day, larger syncytia were present (fig. 3) and the cultures began to develop a "moth-eaten" appearance (fig. 4) similar to that observed in rinderpest-infected prescription-bottle cultures. This can be attributed to necrosis and loss of individual cells and syncytia. By the 16th day, all the smaller cells had left the glass, and only a few of the large stellate cells remained. These also were developing into syncytia, and indicated that they, as well as the smaller cells, were susceptible to the virus. After 28 days no cells remained on the glass.

At this point, it was felt that sufficient knowledge of the viral action had been obtained to proceed with the microcinematographic study. Individual cells undergoing degeneration underwent arborization (shrinking of the cytoplasm with development of streamers) and finally "boiled" before detaching from the glass. In that respect, the effect of the virus could not be distinguished from that of foot-and-mouth virus. The distinctive cell syncytia were formed by fusion of neighboring individual cells rather than by nuclear division without cytoplasmic separation. This had already been postulated by other scientists (1). All syncytia that formed eventually became necrotic and detached from the glass. The "moth-eaten" appearance of cultures with advanced cytopathic effect was caused by a combination of cell loss and tensions resulting from cohesion of remaining viable cells. In these cultures simultaneous cell necrosis and cell regeneration (mitosis) were frequently seen. Rinderpest virus eventually destroyed all cellular elements in the cultures, i.e., both the predominant smaller cells and the large stellate cells. Cell survival and persistent infection of the cell culture, as observed with foot-and-mouth-disease virus, did not occur.

3/ Numbers in parentheses refer to Literature Cited at the end of this paper.

These observations seemed to have only academic value until they were related to another phase of the study. Work with fluorescent antibody had already been tried and had been discontinued because of negative or inconclusive results. The technique had been tried on coverslip cultures showing advanced cytopathic effect (12 to 16 days after infection) with the idea that this would be the optimal time to demonstrate virus in the cells. Upon observing that virus-induced changes were present in the Leighton tube cultures as early as 6 days after infection, work with fluorescent antibody was resumed using coverslip cultures harvested from 1 to 10 days after infection. Virus was demonstrated in the cytoplasm of a few cells as early as the second day after infection. Eight days after infection many cells, including syncytia, showed the apple-green cytoplasmic fluorescence indicative of virus (fig. 5). At this time, cytopathic effect was first evident by conventional microscopic examination of infected prescription- bottle cultures. This technique provides another tool that might be used to study the recently reported observation (2) that rinderpest virus develops in the mitochondria of infected cells.

Observations on the pathology of rinderpest in cattle also can be used to illustrate the importance of method in morphological studies. In this instance, however, the perspective of the pathologist is as important as is the mode of operation. Rinderpest has been defined as a fibrinonecrotic inflammation of the digestive mucous membranes, and as a.....disease of cattle, characterized by.....inflammatory and necrotic changes in the mucous membranes.

In a recent article on rinderpest (3), the authors had the courage to eliminate some of the conventional verbiage that often clutters pathology reports. They did not bring inflammation or inflammatory reaction into the discussion, yet detailed and precise descriptions of both gross and microscopic pathologic changes are given. Truly, the typical gross alterations of the intestinal mucosa can be classified as severe hemmorrhagic enteritis with necrosis and sloughing of Peyer's patches. On the other hand, emphasis on an inflamnatory classification in the histopathologic report would deemphasize the basic lesions of the disease and obscure their significance. These characteristic changes are virus-induced necrosis of columnar epithelial cells covering the mucosa and destruction of lymphocytes in the lymphoid nodules (3). These basic alterations are responsible for the febrile reaction, depression, diarrhea, and the pronounced circulatory changes grossly visible in the gastrointestinal mucosa. Circumstance can assist in this mental process. In any obviously congested and hemorrhagic mucosa, the increase of blood is much less evident in thin histologic sections viewed under high magnification than in the gross. Furthermore, grossly evident circulatory changes need no histologic confirmation. The point in making microscopic examination is to discover why these changes occur. The fact that the authors eliminated conventional references to inflammation and inflammatory reaction illustrates a modern trend among pathologists to focus attention on basic alterations with subsequent analysis and interpretation rather than to simply describe and classify pathological alterations.

On the basis of personal observations, more detailed comment on the histopathology of rinderpest can be made. The lining of the intestional glands is affected equally as much as the surface epithelium. Necrotic lining cells slough into the glandular lumina, and the viable cells that remain elongate to squamous proportions to cover the basement membrane of the glands (fig. 6). In severely affected glands, all the cells become necrotic, slough, and are extruded into the intestinal lumen. The mucosal stroma collapses, the space occupied by the glands disappears, and the glands are permanently lost. In some areas, the mucosa degenerates into a glandless band of stroma (fig. 7) that could have little physiologic function in digestion or absorption. This train of events helps explain the high mortality and the severe symptoms that occur after the febrile reaction and after the virus presumably has ecerted its maximum effect on the epithelial cells.

Interpretive method in the pathological investigation of rinderpest has produced an understanding of the pathogenesis and has brought questions to stimulate research. Some questions concern the nature of the lymphocytic destruction. For example, does virus actually propagate in the lymphocytes and destroy them or could an antigen-antibody reaction on their surface be responsible? On the other hand, is the lymphocytic destruction a nonspecific reaction to "stress"? The similarity of the glandular damage in rinderpest to that in bovine mucosal disease and in feline panleucopenia raised another question. The anatomical effects of the agents of these three diseases are remarkably similar, although no antigenic relationship has been demonstrated. Thus, interpretive method applied to pathological studies can abbreviate the reports and increase the harvest of information and ideas.

Unexpected returns can accrue from work reluctantly undertaken because the prospective harvest appears meager compared with the time and effort required. Initial work with microcinematography at the Plum Island Animal Disease Laboratory was done with tissue culture monolayers infected with foot-and-mouth disease virus.

It was observed that some cells in the cultures failed to develop the typical cytopathic effect. The possible significance of this observation was not immediately appreciated. Attention was focused on the cells that died--the subjects for microcinematography. Finally the question came to mind as to what would happen if cultures at peak cytopathic effect were not discarded as usual but were kept and cared for with appropriate changes of medium? The cultures grew out after several days or weeks (fig. 8) and virus, in low titer (approximately 10^3 TC ID_{50}), persisted in the medium. Furthermore, the regenerated cultures were resistant to superinfection with other types of foot-and-mouth disease virus and to vesicular stomatitis virus. This simple observation has initiated a new line of investigation that deals with persistent infection of tissue culture with foot-and-mouth disease virus and with changes that might occur in virus propagated in that manner.

These comments and observations have dealt with the role of comprehensive method and modern perspective in the study of morphologic alterations caused by viruses. The illustrations have been limited. Examples in such fields as histochemistry, electron microscopy, and experimental pathology have not been included.

Although comprehensive method is important in morphological research, it would not be appropriate to close without drawing attention to the role of routine microscopic examination in the diagnosis of viral diseases. The diagnostic significance of viral inclusions in rabies, canine distemper, and infectious canine hepatitis is well known. Papular stomatitis of cattle, infectious bovine rhinotracheitis, infectious pustular vulvovaginitis of cattle, equine viral rhinopneumonitis, Aujeszky's disease of cattle and swine, and the pox diseases of animals are examples of viral diseases in which the presence of inclusion bodies can be helpful in microscopic diagnosis. In virus diseases like canine distemper, equine encephalomyelitis, hog cholera, and avian epidemic tremor the characteristic histopathology of the brain is an aid in diagnosis. Routine histopathologic examination also is useful in the diagnosis of bacterial, mycotic, toxic, and nutritional diseases. Present methods should not be taken as an excuse by pathologists to rest on their laurels. Advances in biology are being made by biochemists, microbiologists, immunologists, geneticists, and others. These advances present a challenge. The morphologist's future lies in helping to relate this new information to the cell.

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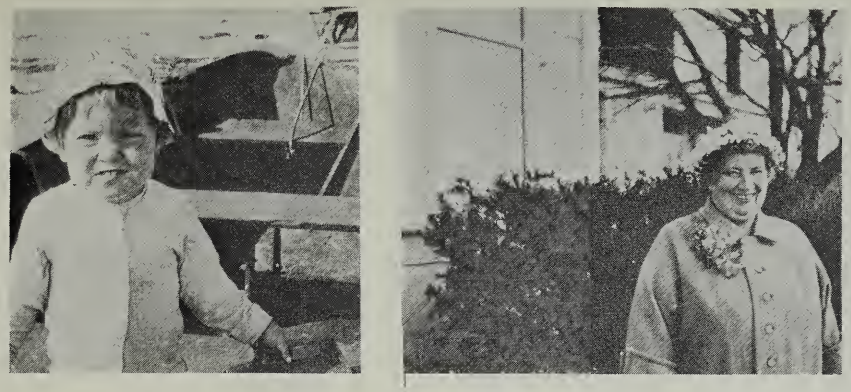


Figure 1.--Sample photographs for a theoretical situation in the study of individuals to illustrate the role of method in morphological research.

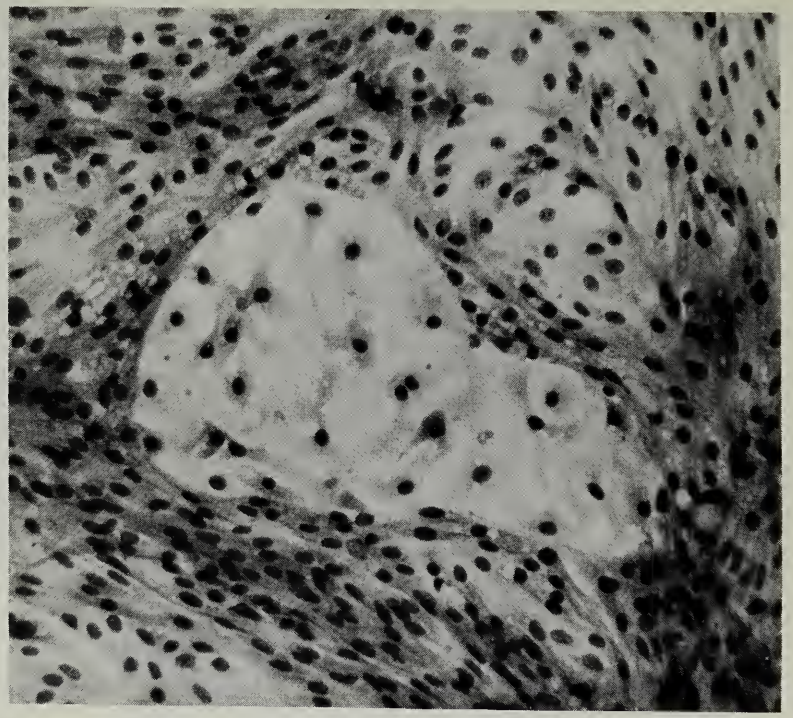


Figure 2.--Island of large stellate cells (histiocytes) in calf kidney cell culture. H & E stain. X 210.

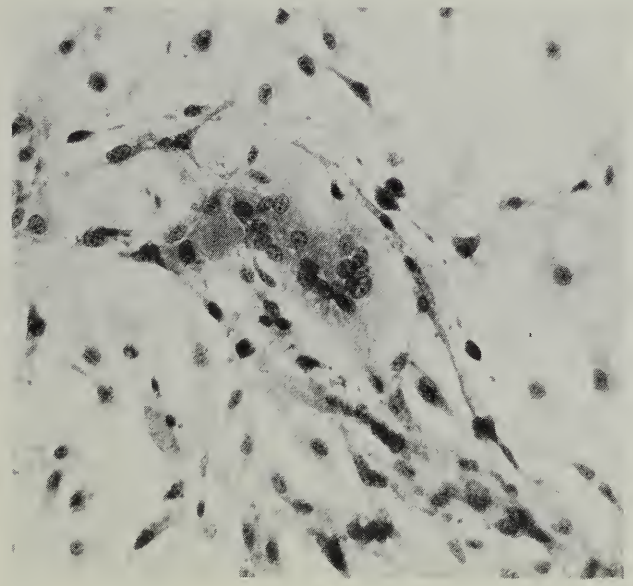


Figure 3.--Typical syncytium in primary calf kidney cell culture 8 days after infection with rinderpest virus. H & E stain. X 210.

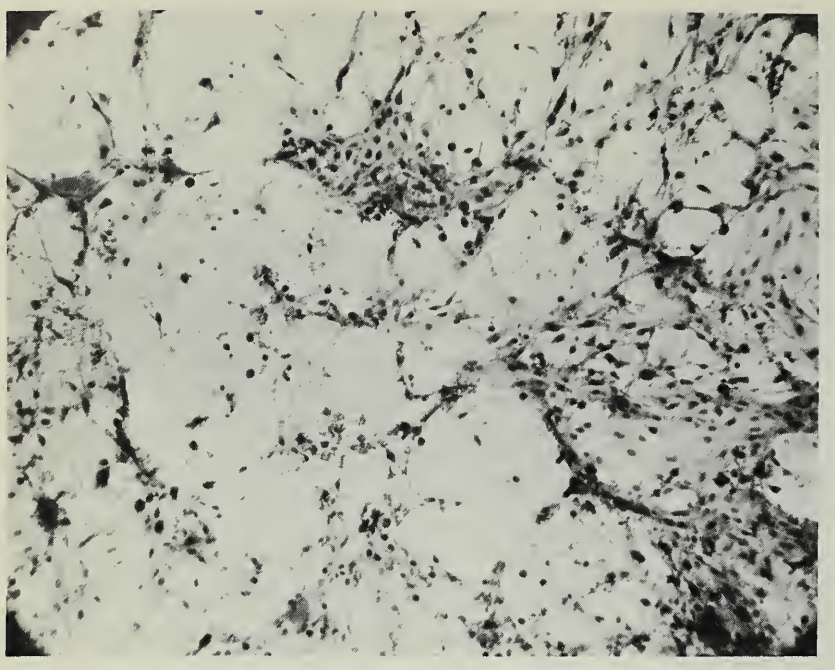
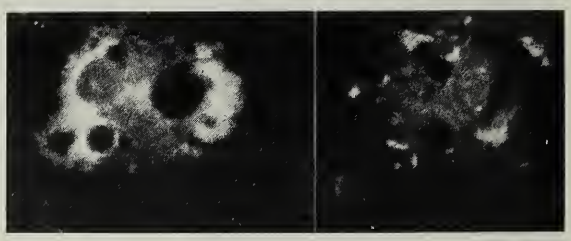


Figure 4.--Primary calf kidney cell culture 8 days after infection with rinderpest virus. Loss of cells has produced the typical "moth-eaten" appearance of a rinderpest-infected culture. H & E stain. X 100.

Figure 5.--Two syncytia in rinderpest-infected primary calf-kidney cell culture. Application of fluorescent antibody technique has resulted in fluorescence at site of antigen in cytoplasm of the cells. Fluorescent antibody staining. X 210.



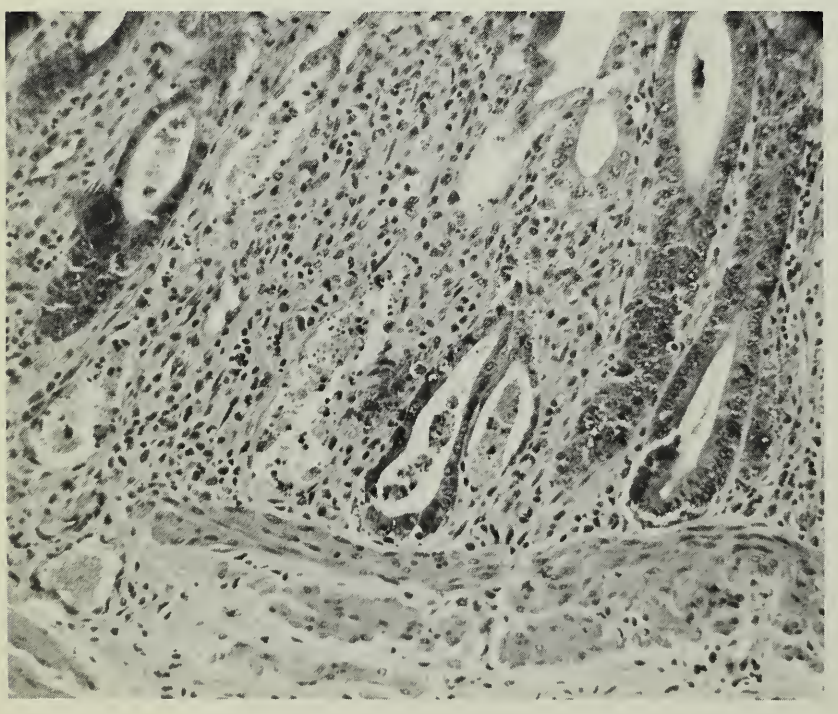


Figure 6.--Histologic section of large intestine of rinderpest-infected steer. The epithelial lining of some intestinal glands has undergone necrosis and partial to complete sloughing. H & E stain. X 210.

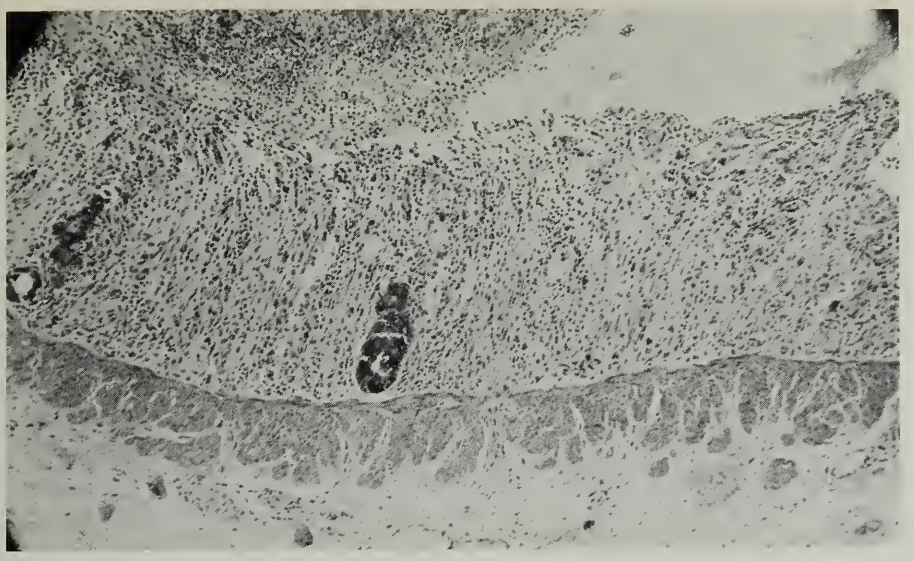


Figure 7.--Histologic section of large intestine of rinderpest-infected steer. Necrosis of the glandular epithelium has resulted in almost complete loss of glands. H & E stain. X 100.

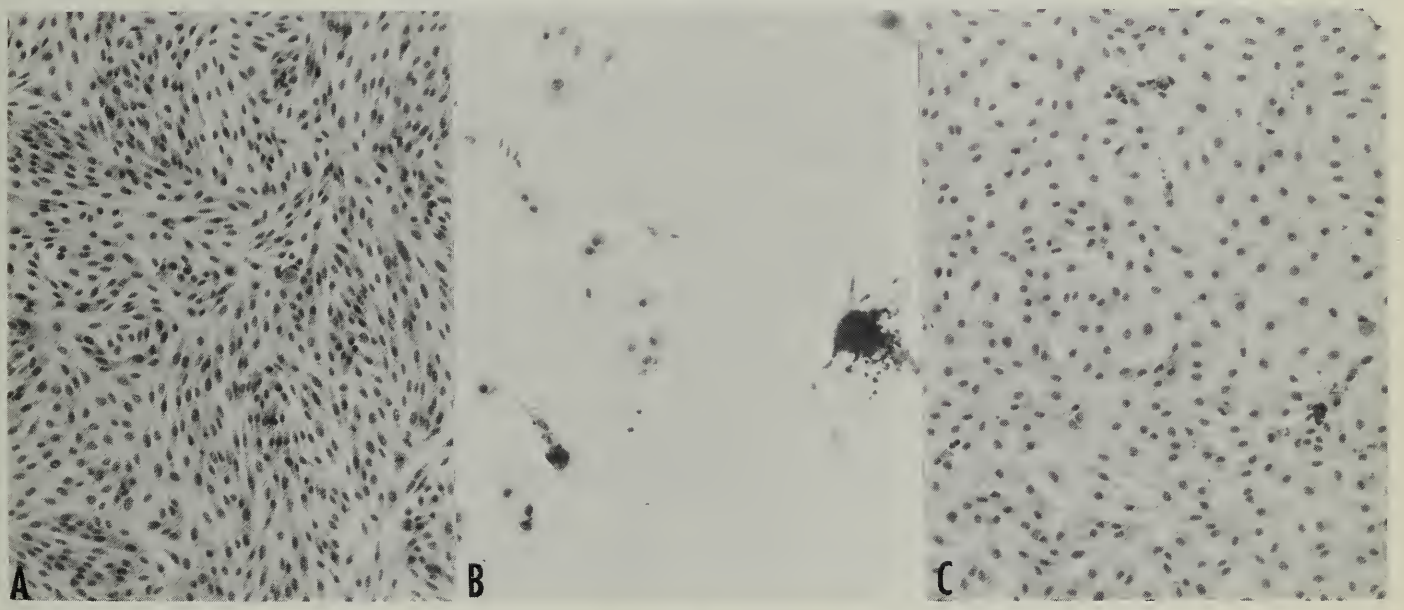


Figure 8.--Cell survival in primary calf kidney cell cultures infected with foot-and-mouth disease virus, type A, strain 119: A, infected control culture harvested simultaneously with B; B, culture 24 hours after infection. Action of the virus has resulted in almost complete loss of cells; C, culture 6 days after infection. The surviving cells have proliferated, and there is almost complete reconstitution of the monolayer. H & E stain. X 130.

RELATION OF SOME SERUM INHIBITORS TO THE CELL RECEPTORS FOR POLYOMA VIRUSES

W. Wilbur Ackermann, Ph. D. 1/

This review partially serves to exemplify the detail to which host-virus relations are currently being subjected. Although it is retelling old material, some observations not previously reported are included. Consideration will be restricted to the first stages in the encounter of virus and host cell. It is primarily a study of the surface reactions of polyoma virus.

The natural host of this virus is probably the mouse, but it infects other species such as the hamster and rat and possibly the guinea pig and rabbit. In all of these species, inoculation results in tumor formation (1).2/ It has been suggested that the virus is a member of a group that includes rabbit papilloma virus and the simian vacuolating agent, SV_{40} (2).

Polyoma virus is obtained easily from tissue cultures in a high degree of purity. Figure 1 is a micrograph prepared in our laboratory using phosphotungstic acid in a negative staining procedure. The agent has a diameter of 48 to 50μ. Like other enteroviruses, it is surrounded by a capsid composed of small capsomeres. It has been estimated that there are 42 of these subunits in the surface (3,4). Whereas several of the enteroviruses (poliomyelitis or Coxsackie) contain ribonucleic acid, polyoma is believed to contain deoxyribonucleic acid. Polyoma agglutinates erythrocytes, as do some enteric agents, but in a manner clearly different. Its behavior more closely resembles that of the myxoviruses.

1/ Department of Epidemiology and Virus Laboratory, School of Public Health, University of Michigan, Ann Arbor, Mich. Many of the studies reported here were aided by a grant from the National Foundation. All electron micrographs were prepared by T. F. Beals.

2/ Numbers in parentheses refer to Literature Cited at the end of this paper.

Figure 2 is a micrograph of an Asian type A influenza virus. Again the use of phosphotungstic acid has delineated some of the fine detail of its surface structure. The virus is surrounded by a sort of ring composed of a brush of spikes. Better micrographs exist. This particular specimen was severely treated with periodic acid before being prepared for electron microscopy. It is remarkable that much of the morphology has not changed. However, its hemagglutination properties have been altered.

Normally, this agent hemagglutinates erythrocytes by combining with a special cellular receptor, a glycoprotein, containing neuraminic acid in the terminal group of its carbohydrate. Located on the surface of the virus, possible in the little brush structures, is an enzyme, neuraminidase. At 22° C. this enzyme can split the neuraminic acid from the receptor. The virus then elutes from the cells and they then no longer agglutinate. The cell is permanently altered while the virus remains unchanged.

In contrast polyoma virus reacts reversibly with suspensions of erythrocytes to form a complex, which will settle from the fluid phase in a typical agglutination pattern. At low temperature (4°),

$$V + C \underset{22^\circ}{\overset{4^\circ}{\rightleftharpoons}} V\,C$$

the complex is stable. The virus can be removed from the fluid because it remains attached to the cell surface. As the temperature is raised from 4° to 22°, the complex dissociates, releasing free virus and unaggluninated cells. The cells and virus remain unaltered, and by merely reducing the temperature the equilibrium can be shifted again toward the complex restoring the typical agglutination pattern (5).(However, occasionally in our laboratory, preparations of the virus produce hemagglutination patterns stable at 22°. Factors effecting this phenomenon have not been analyzed.)

Thus the behavior of polyoma virus is distinct from that of the myxoviruses. In the latter, hemagglutination occurs at either 22° or 4° but has only transient stability at the higher temperature as the complex dissociates spontaneously and irreversibly. Once influenza virus has been treated with periodic acid, it still agglutinates erythrocytes, but the complex does not dissociate spontaneously at 22° with concomitant distruction of cell receptors.

Polyoma and influenza viruses behave differently with the same erythrocyte, possibly because of differences in the viral reactive groups, which participate in the hemagglutination reaction. But it is also possible that multiple types of receptors exist at the cell surface, and that less specific properties of the whole virus particle may be steric or electric influences

select the kind of cell receptor which is to participate. If the latter properties of these viruses differ, distinctly different hemagglutination reactions may be produced by essentially similar viral reactive groups acting upon distinct cell-surface groups. The experiments described next bear upon an analysis of this problem.

Erythrocytes treated with neuraminidase (isolated from cholera vibrio) or with periodic acid, which oxidizes certain glycoproteins, can no longer be agglutinated by either virus. Once the red cell is treated with influenza virus and the latter allowed to elute, the cell cannot be agglutinated by polyoma virus. The receptors of polyoma virus can be destroyed by influenza virus (5). These observations together suggest that the receptors of polyoma virus are glycoproteins. To determine the extent to which cell receptors may react in common with the two agents, the number of reactive sites per cell for each was estimated. Secondly, the ability of the viruses to exclude each other was examined.

An indication of the number of reactive sites was obtained from the saturation numbers of the viruses (i.e., the ratio of amounts of virus required just to produce agglutination of a standard number of cells to the maximum which can be adsorbed). The adsorptions were performed at 4° using various initial concentrations of both polyoma virus and the PR8 strain of influenza virus heated at 56° for 30 minutes. In figure 3 the number of hemagglutination units adsorbed are plotted against the concentration of virus to which red cells were exposed. Adsorption values obtained are seen to be a function of the virus concentration used, since even with low concentrations, virus uptake is never complete. Adsorption values for polyoma virus are always less than those for influenza, and, with increasing concentrations of virus, the adsorption curves become increasingly divergent. Saturation of red cells with polyoma virus occurs after exposure to a concentration of approximately 450 hemagglutinating units, while maximum adsorption of influenza virus does not appear to be obtained with the concentrations used (6).

Further studies have indicated that the receptors for many strains of influenza virus, which are assumed to be finite in number, are increasingly less available as the adsorbed virus is increased. Thus, higher concentrations of virus are required to increase further the receptors bound at the surface. This situation is best described quantitatively by a simply Freundlich isotherm.

To test the capacity of each virus to exclude the adsorption of the other, RBC (red blood cells) exposed to various concentrations of virus, as described in the preceding experiment, were tested for their ability to adsorb influenza virus if the first adsorption was with polyoma virus or were tested for ability to adsorb polyoma virus if the first adsorption was with influenza virus. The results (fig. 4) indicate that complete

exclusion of one virus by prior adsorption of the other is not reciprocal. The prior adsorption of increasing concentrations of influenza virus reduces the capacity of RBC to adsorb polyoma virus, and the adsorption of 6,062 HAU (hemagglutinating units) or more of influenza virus completely excludes the adsorption of any detectable polyoma hemagglutinin. Adsorption of increasing concentrations of polyoma virus onto RBC also reduces the amount of influenza virus that can be adsorbed. However, maximum adsorption to RBC of polyoma virus does not completely exclude the uptake of influenza hemagglutinin (6).

The ability of the viruses to produce mutual exclusion and for influenza to completely destroy receptors for polyoma virus indicate that they react with certain common receptors. The inability of polyoma virus to completely exclude influenza virus shows that some receptors are in the exclusive domain of the latter. On this basis one may propose two classes of receptor--one that can react with either virus and a second that reacts with influenza virus but not with polyoma virus. Each receptor contains an essential component, which is a mucoprotein, because severe treatment with neuraminidase or periodate will destroy both.

Such a proposal is consistent with the large differences in the saturation numbers of the viruses. The number of receptors which can react with each is different. There are clearly more reactive sites for influenza virus than for polyoma virus.

The basis of the differing hemagglutination reactions of these viruses might be assigned either to the quality of their reactive groups or to the nature of the receptor involved, if the temperature stability of the complexes they form at the first receptor could be compared. Unfortunately observation of the influenza complex is confused by the presence of the second receptor.

The question is whether the reaction of polyoma virus with mucoproteins is fundamentally different from that of myxoviruses or whether the receptor available to polyoma virus is fundamentally different from that available to myxoviruses. To isolate and separate these receptor substances from the cell surface and to resolve the problem is a formidable task. However, it was reasoned that clarification might be obtained by study of a mucoprotein recently isolated from bovine serum.

It is well known that there are substances in saliva, blood, urine, and other body fluids that are mucoprotein in nature and which react with viruses in a manner so analogous to the receptor materials that they are referred to as soluble receptors. Normal blood sera of most animal species are capable of modifying the behavior of polyoma virus. Particularly, bovine serum at high dilution has been found to neutralize viral infectivity as well as to block hemagglutination. Particular attention has been called to the latter activity by Deinhardt and others (7). The mucoprotein responsible for the

activity has recently been isolated, and the nature of the complexes it forms with the various myxoviruses and the polyoma virus have been compared (8, 9).

As isolated from bovine serum by adsorption and elution from bentonite, the inhibitor is not homogeneous but appears to be a single polydispersed material. Electron micrographs reveal that the inhibitor has a platelike structure of uniform thickness (10 to 15 mμ.) and of variable size defined in two dimensions by rather sharp linear boundaries. There is little tendency for it to fold onto itself or to aggregate in clumps (fig. 5).

Dilution of an active bovine serum and centrifugation did not yield the structures obtained by adsorption to and elution from bentonite. However, if the purified inhibitor is added to serum, it can be recovered by centrifugation. It is speculated that the material is pure but is capable of aggregation in two dimensions to yield platelike structures of uniform thickness but of variable size. Presumably aggregation occurs as the material is isolated and concentrated. Efforts so far to disperse the platelike structures into a single homogeneous subunit have been unsuccessful.

The stability of the aggregate in water, in physiologic saline, and in blood serum is what one would expect also of the limiting structure of a cell surface. The spontaneous formation of these structures during the isolation procedure may be analogous to the formation in cells of reticulate structure. That is, the latter may result simply as a consequence of the intrinsic properties of the subunits of which they are composed, and the properties may be the capability of two-dimensional aggregation.

The serum inhibitor contains 10.4 percent nitrogen by the Koch-McMeekin method; 0.38 percent sialic acid by the Aminoff method; and 3.1 percent by the Ehrlich method; protein by the Lowry method; but no phosphorous according to the Fiske-SubbaRow method. These data and the sensitivity of the biologic activity to trypsin, neuraminidase, periodic acid, and the stability to heat (100° for 30 minutes) and dialysis indicate it is a glycoprotein.

In a standard test the material will inhibit the hemagglutination of guinea pig erythrocytes produced by two units of polyoma virus when present at a concentration of one part per million by weight.

Studies of the order of addition of the three reactants--red cells, polyoma virus, and inhibitor--suggest that the basis of inhibition is the interaction of virus and inhibitor to form a complex that is incapable of agglutinating red cells. The complex is stable only at 4° and dissociates in a reversible manner at higher temperatures (22°). From their behavior there is no evidence that the three reactants produce any irreversible alteration of each other. The character of this complex recalls that found between the virus and red cell described above. However, the rate of reaction between virus and inhibitor appears to be less rapid and dependent upon the concentration of both virus and inhibitor.

Demonstration of the full capacity of the mucoprotein to inhibit requires a long period of preincubation with virus prior to addition of red cells.

$$V + I \underset{22^\circ}{\overset{4^\circ}{\rightleftharpoons}} (VI)$$

$$V + C \underset{22^\circ}{\overset{4^\circ}{\rightleftharpoons}} (VC)$$

In figure 6 is a theoretic curve representing an equation derived from one that expresses a second order reaction. A reaction rate constant, which closely fits the experimental results, was calculated from this equation, which is expressed as follows:

$$k = \frac{2.3}{t} \cdot \frac{1}{I - V_o} \cdot \log \frac{V(I - V)}{I(V_o - V_t)}$$

in which t is the time in hours of preincubation of virus with inhibitor which just produced inhibition; I is the initial inhibitor concentration expressed in units of hemagglutination inhibition; V_o is the initial virus concentration of two hemagglutination units; V_t is the concentration of virus at time t which just fails to give a detectable hemagglutination reaction, assumed value being 0.8 hemagglutination units. The value of k used to plot the theoretic curve is 0.004 and produced a reasonable fit to the data.

In table 1 are the hemagglutination inhibition titers obtained with a preparation of serum inhibitor using a variety of hemagglutinating viruses. These were preincubated with inhibitor at either 4° or 37° prior to addition of red cells. Also indicated is the temperature at which each agent agglutinates the species of erythrocyte used in the particular test. All the viruses hemagglutinate at 4°, but of these only mumps virus, polyoma virus, and the PR8, A/57, swine, and Tokyo B strains of influenza virus react at this temperature with inhibitor. Furthermore, they differ significantly in maximum dilution of inhibitor to which they respond, and this is unrelated to whether or not they are treated with KIO_4. Of the inhibitor-sensitive agents, only A/57 and swine produced stable hemagglutination at 37°, and likewise only these strains reacted with inhibitor on preincubation at this temperature. These patterns were stable when shaken and allowed to reform at 22°.

Table 1--Specificity of the hemagglutination inhibitor (HAI) of polyoma virus for various hemagglutinating viruses

Virus	HAI titer [1] Preincubated with virus		Hemagglutination characteristics [2]		
	4° C.	22° C.	4° C.	22° C.	37° C.
Polyoma	192	0	+	-	-
Influenza strains[3]:					
PR8	96	0	+	+	± [4]
A/53	0	0	+	+	-
A/57	48	64	+	+	+
Swine	64	192	+	+	+
Lee	0	0	+	+	-
Tokyo B	96	ND [5]	+	+	ND
C/JJ	0	ND	+	+	ND
Sendai	0	ND	+	+	ND
Mumps	16	ND	+	+	ND
NDV[3]	0	0	+	+	± [4]
ECHO (3,9,10,11)	0	ND	+	+	ND

1/ Titers expressed as the reciprocal of dilution just producing an incomplete shield.

2/ Human O cells were used for tests with ECHO viruses; chick cells were used with type C influenza, Tokyo B, and mumps; guinea pig cells were used with all others.

3/ Strains PR8, A/53, A/57, swine, Lee, and Newcastle disease (NDV) were converted to the indicator state with KIO_4.

4/ Occasional spontaneous loss of hemagglutination titer occurred when compared with titration at 4° C.

5/ ND means tests not done.

The viruses which do react with the isolated mucoprotein show the same temperature requirements as their corresponding hemagglutination reactions. The KIO_4-treated PR8 strain of virus was found to be analogous to polyoma virus, in that positive hemagglutination patterns produced by it at 4° became negative at 37° but subsequently reformed at 4° without loss of titer. The treated PR8 strain was found also to react reversibly with the isolated mucoprotein, combining at 4° and dissociating at 37°, thus demonstrating a further analogy between the viruses and between the red cell receptor and the isolated mucoprotein.

Since the mucoprotein can be visualized in the electron microscope where its characteristic morphology is easily recognized, it should be possible to detect the hypothetical complex of virus and inhibitor. The stability of the complex formed between the inhibitor and the periodate-treated A/57 strain of influenza virus was found particularly ideal for this purpose. Figure 7 is an electron micrograph of such a mixture, shadowed with palladium and showing a number of characteristic virus particles adhering to the surface of the platelike structure, which appears distorted out of plane as if there were more than one point of attachment between the virus and the mucoprotein. Negatively stained preparations showing the characteristic substructure of the influenza virus leave no doubt as to the identity of the virus in such aggregates.

Certain hemagglutinating enteric viruses (ECHO 3,9,10,11), whose cell receptors are presumed not to be mucoproteins of the neuraminic acid type, gave no evidence of interaction with the isolated mucoprotein inhibitor of polyoma virus. Viruses like the polyoma virus, also contain no neuraminidase. The representative myxoviruses considered here, which do react with mucoproteins of the erythrocyte surface, are all sensitive to the complex of inhibitors in bovine serum. But in contrast, they show marked characteristic reactivities in their reaction with the single mucoprotein isolated from the serum. The peculiar ability of polyoma virus to agglutinate erythrocytes and elute from them without enzyme action seems clearly a consequence of the unusual property of the complex it forms with mucoprotein rather than an unusual mucoprotein available to it and with which other viruses do not react.

The relationships between certain of these agents, indicated by their reactivity with the mucoprotein, are not revealed by antigenic analyses which also describe surface character. The Lee strain of type B and A/53 do not react, whereas Tokyo B and A/57 do. Neither the surface nor internal antigens of these pairs are known to be related immunologically. The use of isolated mucoproteins as reagents for the classification of viruses may demonstrate relationships of greater biologic significance than the use of antigenicity, so important in immunity.

It should be emphasized, however, that knowledge of the reactive chemical moiety of the erythrocyte, which these viruses share, contributes little to understanding the infection of host cells by polyoma virus. For this agent, unlike the myxoviruses, the red cell is not a useful model. Host-cell receptors of polyoma virus appear not to be mucoprotein, are distinct from those of influenza virus, and presumably operate at 37° at which

temperature the red cell receptor complex is unstable (6). Analogous to the host-cell receptor is a different serum component. At 22° it acts upon virus to block infectivity as well as the hemagglutinating activity (10). This material has been partially purified, and it can be anticipated that it will be also of value in the further study of the reactive surface of polyoma virus.

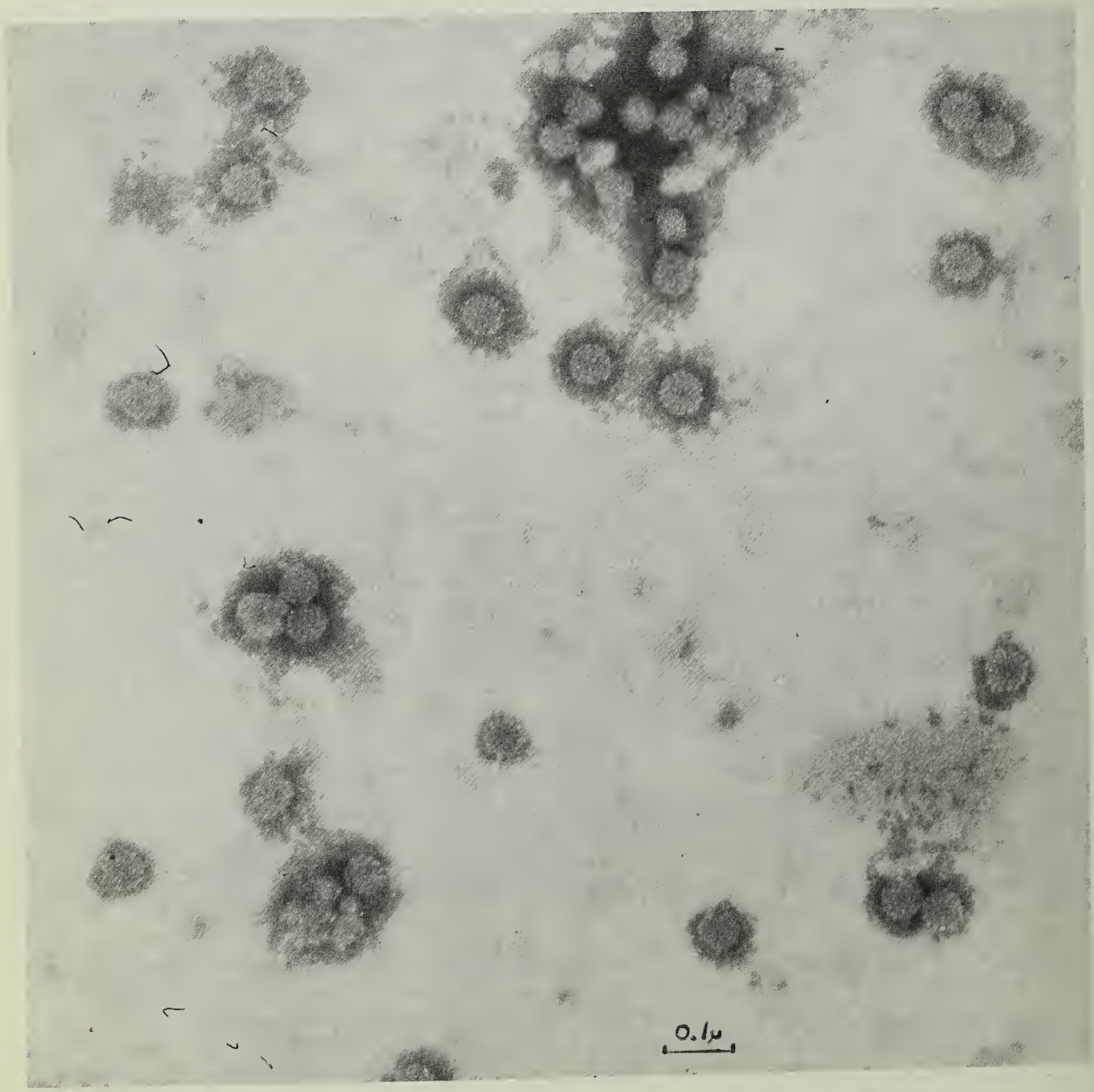


Figure 1.--Preparation of polyoma virus negatively stained with phosphotungstic acid. Magnification by electron microscope, X 124,000.

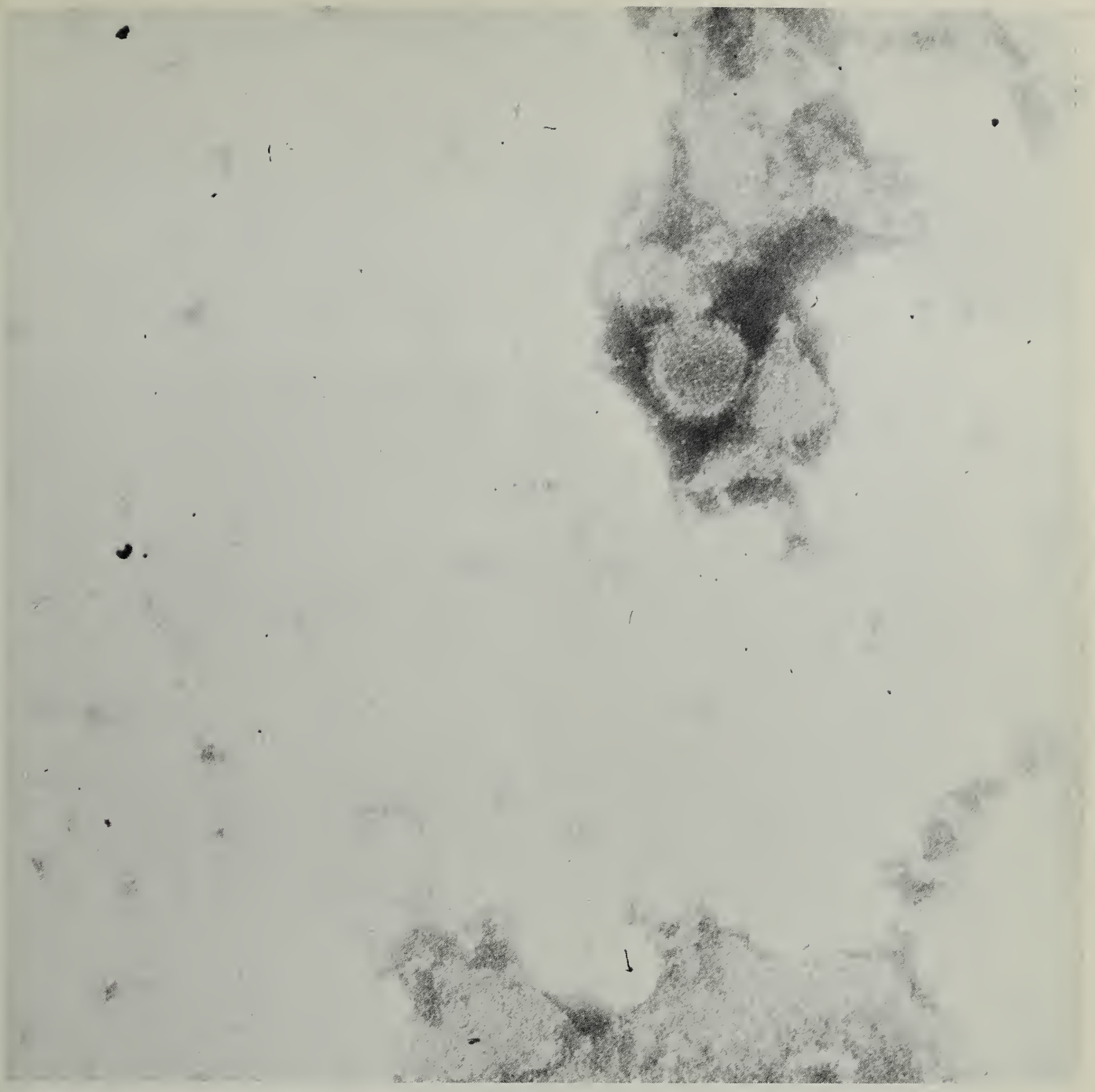


Figure 2.--Influenza virus type A Asian strain inactivated with KIO_4 and stained with phosphotungstic acid using negative staining method. Magnification by electron microscope, X 10,000+.

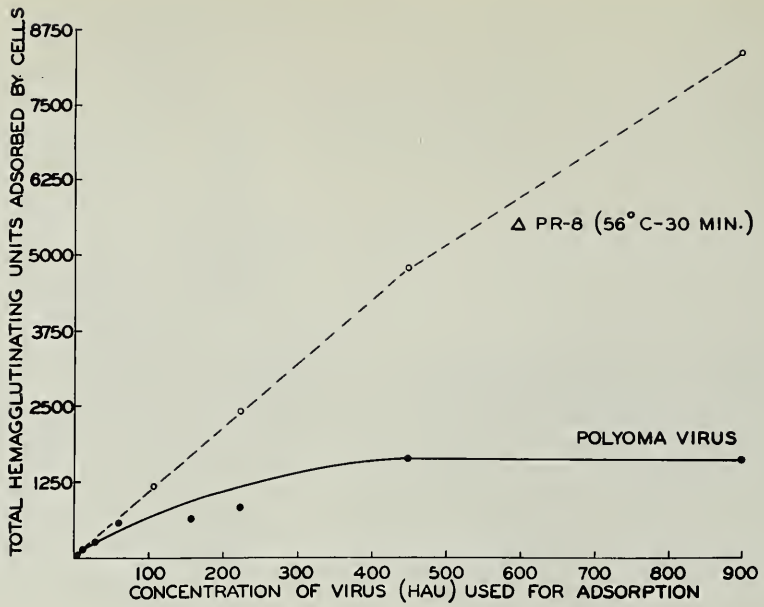


Figure 3.--Effect of virus concentration on the adsorption of polyoma and heated influenza viruses by red blood cells. The concentration of each virus is expressed as the number of hemagglutinating units per 0.4 ml., so that the total virus in 5 ml. used for adsorption is 12.5 times the given value.

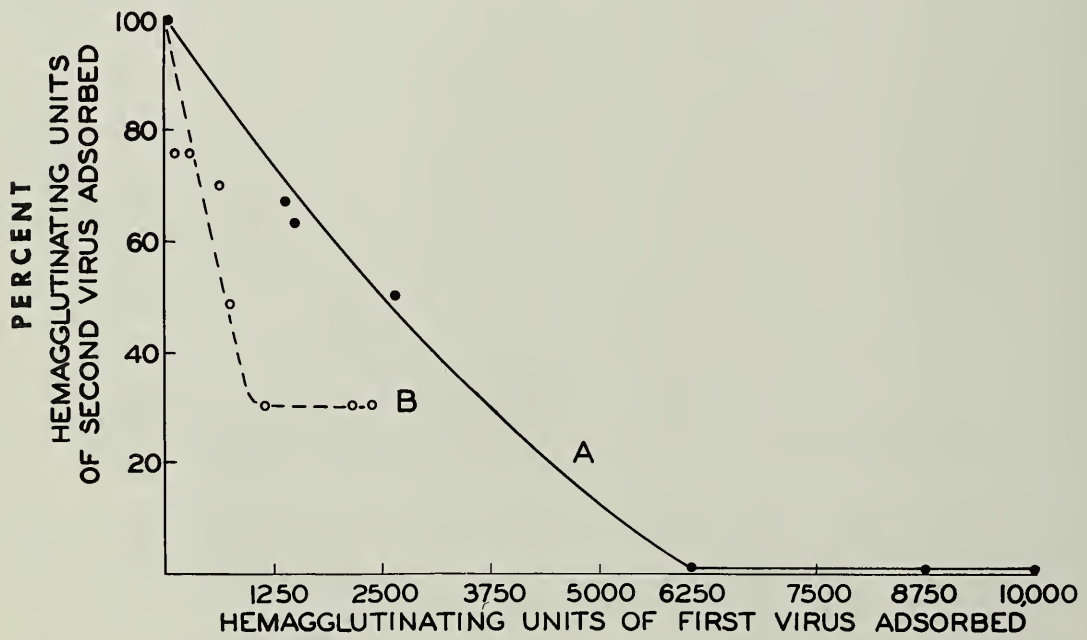


Figure 4.--Reciprocal exclusion between polyoma and heated influenza viruses. Curve A represents the adsorption of polyoma virus by 0.5 ml. of 0.5 percent red blood cells after adsorption of varying amounts of influenza virus and is expressed as percentage of adsorption to untreated cells. Curve B represents the adsorption of influenza virus by 0.5 ml. of 0.5 percent red blood cells after adsorption of varying amounts of polyoma virus and is expressed as percentage of adsorption to untreated cells.

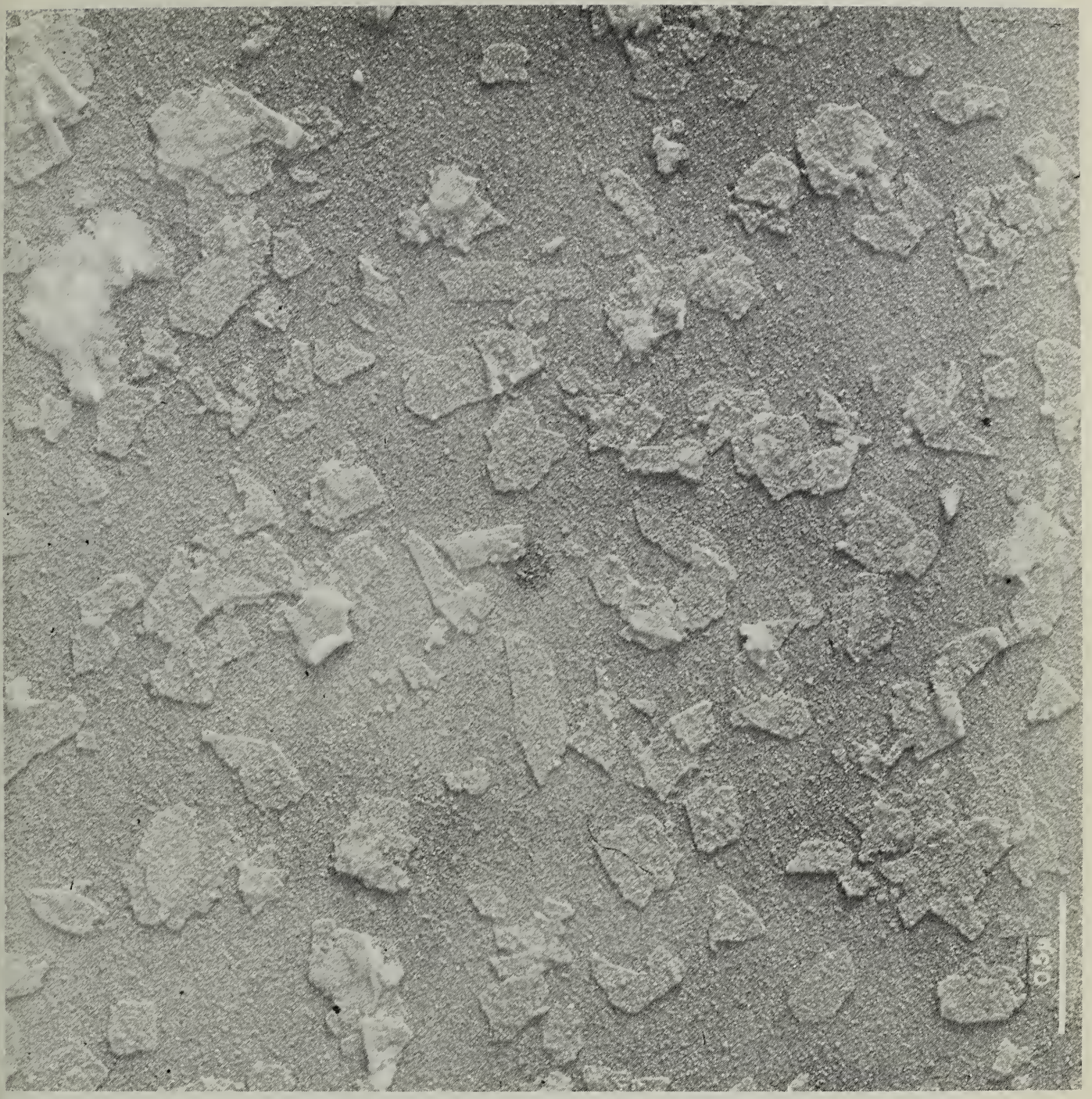


Figure 5.--Micrograph of purified inhibitor deposited on a grid from borate buffer suspension, washed with water, drained, and fixed while moist with osmium tetroxide, washed again, drained, frozen at -72° C., dried, and shadowed with palladium at an arc tangent of 1 to 5 before examination. Magnification by RCA EMU 2-A electron microscope.

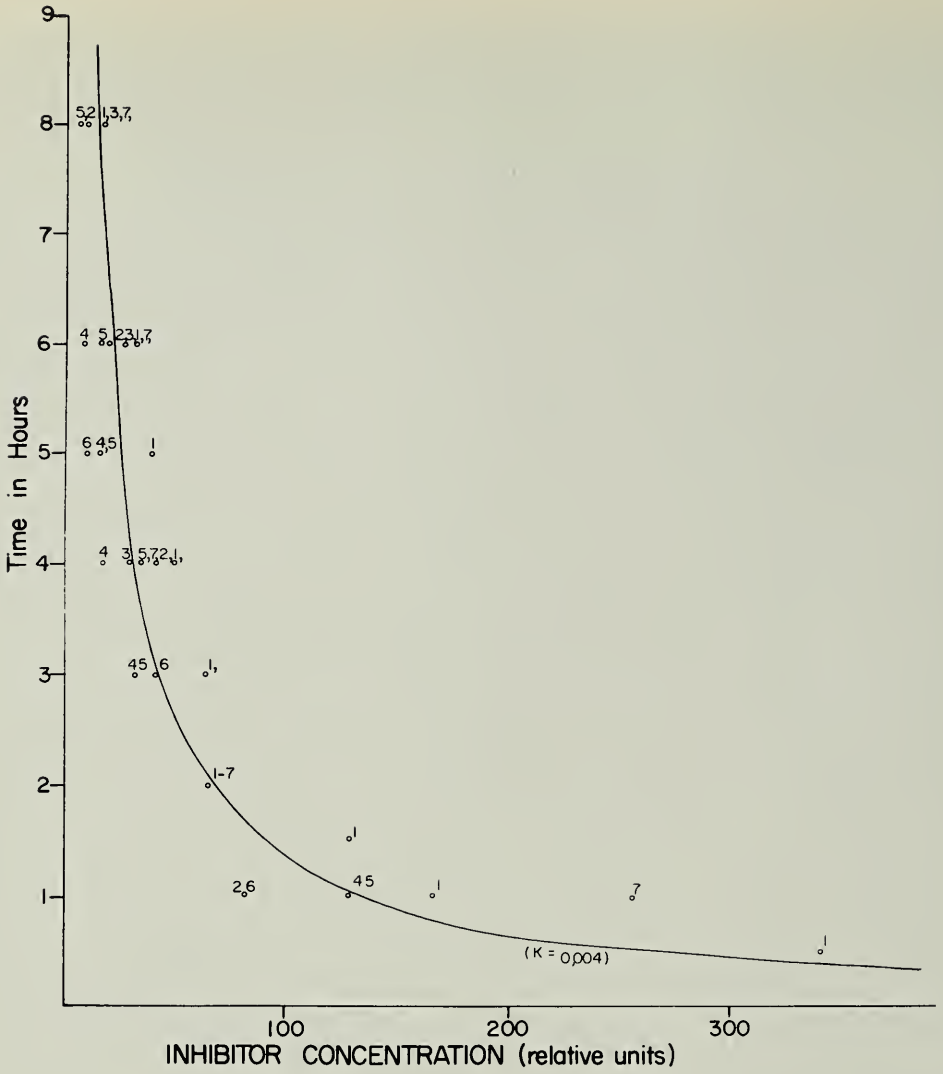


Figure 6.--Rate of reaction of isolated inhibitor from bovine serum with polyoma virus at 4° C. The theoretic curve represents an equation derived from one that expresses a second order reaction.

Figure 7.--Micrograph of a mixture of purified inhibitor with a crude preparation of the A/57 strain of influenza virus, prepared like that in figure 5 and shadowed with palladium.

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HOST CELL INTERACTION WITH THE BAI STRAIN A AVIAN TUMOR VIRUS

Hideo Ishiguro, R. A. Bonar, Ursula Heine, Guy de Thé
Dorothy Beard, and Joseph W. Beard[1/]

Among the most significant advances in virology have been those emanating from studies of host-virus interrelationships at the cellular level. In this field, use of the electron microscope has afforded the means of disclosing not only the ultrastructural aspects of affected cells but much evidence, as well, of many features of intimate associations of the cells with the agents, including morphologic manifestations related to formation and elaboration of the agents. Despite the remarkable findings in many virus diseases, comparatively little progress has been experienced in similar work with the avian virus-induced tumors. Most of the growths examined--Rous sarcoma (1-5)[2/], Fujinami myxosarcoma (6), and Murray-Begg sarcoma (7)--showed but dubious evidence of cell involvement either by ultrastructural changes or in the release of virus by the cells. Some clarification of the problem was effected by demonstration of virus assembly by budding of the cell membrane of ertyhroblasts (8-12) in erythroblastic leukemia, and just recently a similar process was detected in the Rous sarcoma (13, 14). Even in these diseases, however, morphologic evidence of participation of intracellular structures in virus production was not apparent.

In contrast to these results, the recent findings with the neoplasms induced by the BAI strain A virus (15, 16) have been much more revealing of cellular response in the infectious process. The myeloblasts of myeloblastic leukemia exhibit (17-21) cytoplasmic structures specific to the disease and apparently concerned with synthesis and release of the agent. Opportunity for broadening the area of study of analogous relationships of the same agent to other neoplastic cell systems has been afforded by the character of the renal tumor (22-26) induced by the BAI strain A virus. This growth is a mixture of a variety of tissues of different cell types, each exhibiting characteristic features of pathologic response and of cell-virus relationships (25, 26). The kidney tumor thus represents not a single growth but in effect a rather broad spectrum of neoplastic responses available for comparative and correlative investigations. In addition, various attributes of the growth with respect to probable origin and to the characteristics of cell differentiation and organization provide the basis for some speculation relative to the biologic processes contributing to

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1/ This work was performed in the Department of Surgery, Duke University Medical Center, Durham, N.C. Funds were provided through a research grant (C-4572) from the National Cancer Institute, National Institutes of Health, U.S. Public Health Service; through a grant from the American Cancer Society, Inc., (E-84A), and through the Dorothy Beard Research Fund. Hideo Ishiguro is on leave from Yamaguchi University, Shimonoseki, Japan. This paper was presented at the symposium by Joseph W. Beard, M.D.

2/ Numbers in parentheses refer to Literature Cited at the end of this paper.

development of the neoplasm. It is the purpose of this report to consider the findings thus far with myeloblasts and with the renal neoplasm and to consider possible implications concerned with the respective contributions of the host and the virus to the cell-virus interactions.

BAI Strain A Virus

In its morphologic features as determined in electron micrographs of thin sections (27), the BAI strain A virus (fig. 1) is indistinguishable from other strains of avian tumor viruses. Chemically the agent contains 2.17 percent RNA, 9.9 percent nitrogen, 35 percent lipid, 1.27 percent phosphorus, and no DNA (28). It is likely that other chicken tumor viruses are of similar constitution (29, 30).

Unusual properties, however, serve to distinguish the agent as an entity separate and distinct from other avian tumor viruses (16, 31-33). In addition to specific viral antigen demonstrable by neutralization and precipitation with chicken immune serum, the virus particles contain chicken tissue antigen (31), as indicated by neutralization with antichicken protein rabbit immune serum. Forssman antigen is likewise a component (31) of the particles, since serum from rabbits inoculated with homogenates of guinea pig kidney neutralizes the infectious attributes of the virus.

A unique property of the agent is its capacity to dephosphorylate adenosine triphosphate (34-38). The enzyme activity is quantitatively proportional to the number of virus particles, as determined by direct electron microscopic count (39), occurring in the plasma of chickens with myeloblastosis and in the fluid of cultures of myeloblasts derived from the circulating plasma of chickens (40-43) with leukemia or by treatment of normal bone marrow with the agent in vitro (44). ATPase activity is not known to be associated with any other strain of chicken tumor virus, and, in consequence, the enzyme constitutes a basis not only for identification of the agent under suitable conditions but for titration and estimate of particle number or virus mass with relatively great accuracy.

Myelobilastosis

Studies of the BAI strain A have been greatly facilitated by occurrence of the virus in very high concentrations (36), as many as 2 trillion particles per ml, in the blood plasma of some chicks with myeloblastic leukemia. From such birds, the agent can be isolated in quantities of 0.3 mg dry weight per 1 ml. of plasma. The most notable feature of the disease is the occurrence also of myeloblasts in large numbers in the circulating blood plasma (36). The concentrations of the cells reach levels of 2 billion per ml. of plasma and may constitute more than half the total blood volume.3/ The concentration of virus in the blood is statistically related

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3/ In the oral presentation, a comparison photograph was used that is not included in this publication. A thick white layer of myeloblasts, obtained by centrifugating 10 ml. of blood from a chick with myeloblastic leukemia, was compared with thin buffy coat of the same volume of blood from a normal chicken.

to the number of myeloblasts, but this means only that blood of high myeloblast content is more likely to contain much virus than that of low myeloblast content.

Myeloblasts obtained from blood of leukemic chicks (40-43) or derived by treatment of normal bone marrow (44) in vitro grow at exponential rates for indefinite periods in tissue culture. In a culture fluid of 30- to 50-percent chicken serum in medium 199 with glucose in 0.5-percent concentration and folic acid 20-40 μg per 100 ml. the cells double in number in 2.5- to 7-day periods (42, 43). Virus is liberated into the culture fluid at constant rates of approximately 25 to 50 virus particles per cell per hour. Cultures suitable for many purposes of study are prepared by seeding about 2.5×10^7 myeloblasts per milliliter of culture fluid in volumes of 5.0 ml. of medium in 50-ml. Erlenmeyer flasks and incubating the suspension on an oscillating shaker at 37° C. The cultures may be sampled in minute volumes at any time for cell count with a hemocytometer or for virus assay by measurement of ATPase activity (40, 43). Estimate of the enzyme can be made on large numbers of samples with an automatic analyzer (43).

The characteristics of the leukemia with respect to the occurrence and properties of both the myeloblasts and the virus have provided unusual opportunities for investigations of cell-virus interactions with a variety of techniques. Particularly informative results have been obtained by study of the myeloblasts in thin sections with the electron microscope (17, 19) and correlation of the findings with observations made by conventional light and phase microscopy (18-21). The results have given indications of specific and unique relationships of the virus to the cell in the processes of synthesis and liberation of the agent by the myeloblast.

It has been surprising to learn that electron micrographs of thin sections of myeloblasts, fixed immediately after removal from the blood of diseased chicks, show virtually no evidence of significant relationship of virus to the cell (17, 19). One or a few virus particles are occasionally present in small vacuoles, and no virus is present in the cytoplasm. In their ultrastructural aspects, the diseased myeloblasts are indistinguishable from the myeloblasts of normal bone marrow except for the occasional presence of virus particles in vacuoles.

Striking cytoplasmic changes occur, however, when the cells are transferred from the chicken to tissue culture (17, 19). Within 8 to 24 hours and for several days afterward, there appear cytoplasmic structures (fig. 2) obviously specific to the myeloblast diseased with this virus. The structures have the appearance of inclusion-like bodies of variable size and shape. The bodies--viroplasts--are enclosed in a single or sometimes double wall and are filled with a material of relatively high electron density in osmium-fixed specimens. Embedded in random distribution in the substance of the viroplasts are virus particles showing the typical nucleoids (fig. 1). The viroplasts show no special relationship to other cell structures but move about freely throughout the cell as seen in moving pictures of the living element.

After longer periods of culture, there are further and progressive changes in the viroplasts. These result in a series of bodies varying in

structure from viroplasts, such as those shown in figure 2, to other bodies containing more or less internal substance and, finally, to vacuoles. In all stages of the series, one or more virus particles may be evident. The findings have been interpreted to indicate that the viroplasts are loci of virus elaboration in the cell. With synthesis of the agent, the substance of the viroplast is depleted, and the virus is then liberated from the cell by opening of the vacuole-like structure through the cell membrane. In this case it would be assumed that the virus thus formed does not come in contact either with the cell membrane or the cytoplasm. This possibility is supported by lack of evidence of virus or virus precursor in the cytoplasm or of more than very rare buds at the cell membrane.

Thus far it has not been possible to obtain unequivocal evidence of the origin of the viroplasts or of their relationship to other cell structures. Early in the work it was thought (19) that they might represent virus-infected and altered mitochondria. Further study, however, did not support this view but did suggest another possible explanation of the derivation of the viroplasts. Examination of the myeloblasts from culture with the phase contrast microscope revealed (18-21) the presence in the cells of structures which could be correlated and identified with the viroplasts first seen in thin sections. Cytochemical studies showed that the viroplasts, like the virus particles, were strongly positive for ATPase activity. Parallel examinations of cells of the normal bone marrow revealed ATPase activity of the granules of myelocytes and granulocytes.

The findings in these and other similar investigations provided the basis for the hypothesis that viral determinative material becomes integrated at the sites of or in the precursor structures of the normal granules, and that the viroplasts are developed as altered granules with the special function of synthesis of the BAI strain A virus. Unequivocal evidence that such a process occurs has not been obtained. Indirectly it has been apparent, however, that structures of the nature of the viroplasts have not been observed in any cells other than myeloblasts infected with the BAI strain A virus, and granules of normal morphology do not appear in the virus-associated cells. The interrelationships of the virus and myeloblast are specific to this cell type, and elaboration of the virus by other affected elements occurs by the entirely different processes of budding from the cytoplasmic membrane as will be described later.

A high degree of compatibility of cell functions and the processes attending infection of the cell and synthesis of the virus are indicated by continued growth of the myeloblasts in tissue culture. Ultrastructural study reveals no evidence of cell damage other than the presence of viroplasts and the failure of the cell to differentiate to the stages of myelocytes and granulocytes. It is notable in this respect, however, that virus of the characteristics of particles outside the cells do not accumulate in the myeloblasts in culture. Instead, virus synthesized, presumably in the viroplasts, is eliminated continuously and at a constant rate from the cell.

That association of virus with the cell does not notably interfere with the physiologic functions of the cell that are critical for its metabolic processes and growth is evident in electron micrographs of the myeloblast in mitosis. As seen in figure 3, cell division proceeds despite the presence of numerous viroplasts. The micrograph shows, also, one means of transfer of virus from the parent cell to the progeny. It has been speculated (45) that the conversion of normal cells to elements of neoplastic behavior is the result of mutation induced by integration of viral determinative material with the genetic elements of the cell. Further, it has been supposed that virus is distributed to cell progeny by genetic mechanisms. Although there is no proof either for or against this hypothesis, it is certain, as seen in figure 3, that the BAI strain A virus may be transferred from parent to daughter cells simply by mechanical distribution of viroplasts during the process of cell division in the same manner as mitochondria and other cell components are distributed.

Nephroblastoma

Gross and Histologic Pathology.

Interpretations relative to the fundamental principles governing cell growth and the differentiation and organization of the tissues of the nephroblastoma are dependent on concepts of the origin of the growth in the kidneys. A principal manifestation of this virus-induced growth, as well as of analogous tumors of man, Wilms' tumor (46-48), and animals (49-51), is the occurrence of epithelial structures resembling more or less closely all the various segments of the normal nephron. An insight into the processes responsible for these features of the tumor may be gained by consideration of the derivation and factors concerned with development of this normal structure.

The normal nephron (52-55), inclusive of the glomerulus and the tubular system to the junction with the connecting tubule, is a derivative (55-57) of the embryonic nephroblastema. In the development of the kidney, collecting tubules budding from the Wolffian duct push into the renal cortex and into contact with the nephroblastema. Under the influence of inductors (56, 58) associated with the collecting tubules, cells of the nephroblastema undergo epithelial differentiation. Continued growth of the initial group of epithelial cells (56) results in progressive differentiation that gives rise to the various cellular elements constituting the glomerulus, the proximal and distal tubules, and the loops of Henle.

It has been the hypothesis that the nephroblastoma arises from nephrogenic cells, which are residual as groups or as "buds," (fig. 4, A and B) in the postembryonic kidney (46, 48), and there is much to support the view that the virus-induced nephroblastoma likewise originates from such nephrogenic buds. In some instances it appeared that the onset of tumor growth could be traced from such buds. If this were the case, it was thus further evident that the epithelial elements were derived from cells having all of the potentials for growth and differentiation inherent in cells of nephrogenic attributes as exemplified in the most primitive state by the nephroblastema.

It should be recalled that the nephroblastema is of mesenchymal nature; consequently, the epithelial cells of the nephron are derivatives of mesenchyme (57). Tissues of the tumor other than those composed of epithelium--that is, stromal cells, cartilage, and osteoid--are likewise derivatives of mesenchyme. Under both normal and special conditions, including infection with other tumor viruses (59, 60), cartilage and bone originate by differentiation of mesenchyme. It is thus not entirely surprising that all the elements of the renal tumor may be representative of the expression of the natural potentialities for specialized differentiation of cells of fundamentally nephrogenic attributes.

Incidence of the nephroblastoma in the chicken population exposed to the BAI strain A virus is high and may reach levels of 85 percent (23) in dose groups of birds showing no evidence of leukemia. Nevertheless, the number of growths in the individual bird is small; only a single tumor may be found in 50 percent of the chickens with tumor. Some birds have a single tumor in each kidney, and less often two or more tumors may be found in one kidney. This would appear to indicate a high susceptibility of the kidney cells to infection with the agent and a very limited distribution of susceptible cells in the individual bird.

Grossly, the tumors vary from microscopic size to spheroidal masses several inches in diameter (figs. 5, A and B, and 6). All arise in the superficial aspects of the kidney and become pedunculated or attached by a thin pedicle. Most are cystic, some are firm, and others are very firm or hard. There is no invasion of kidney tissue, and no metastases have been found in other organs. The growth is thinly encapsulated in regions away from the kidney, but no definite capsule separates tumor from kidney tissue.

Apparent development from structures like nephrogenic buds in the normal kidney (fig. 7) is illustrated in figures 8 and 9. In these minute growths (figs. 8 to 10), the epithelial elements are arranged in poorly organized tubules and small irregular cysts lying in a rather dense supporting stroma. Little evidence is seen of formations resembling glomerular corpuscles.

In some growths, differentiation and organization of the epithelial elements closely approximated (figs. 11 and 12) the normal in the formation of both glomerular corpuscles and tubules. Especially notable in figure 12 (see also fig. 21) are the capillaries in the glomerular tuft containing red blood cells. Evidence has been advanced (56) to indicate the presence of erythropoietic tissue in the normal glomerular tuft, and the micrographs suggest that such is the case also in the renal neoplasm. Well illustrated, also, in figures 11 and 12 is the stroma of undifferentiated cells of epitheloid or mesenchymal appearance.

Differentiation and organization to these levels are not often seen. Instead, there is great variation from these epithelial arrangements resembling glomeruli and tubules to masses, cords, and sheets of epithelium (figs. 13 and 14). Completely unorganized epithelial cells may merge with spindle cells that have a morphology seemingly transitional between the two cell types.

Extremes of differentiation toward the spindle-cell type results in the occurrence of frank sarcoma (figs. 14 and 15), either as well-circumscribed growths or as growths merging with poorly organized tubular structures.

Occasional tumors (figs. 17 and 18) contain much cartilage and also osteoid, which obviously are derived from the background stroma of incompletely differentiated cells. Transitions of the stromal cells to prechondrocytes and chondrocytes as well as to osteoid cells (19) are clearly evident in figure 19 but are better illustrated in electron micrographs of thin sections (fig. 31).

Ultrastructure.

Ultrastructural studies of the nephroblastoma confirm the details of histologic structure and in addition reveal the finer aspects of differentiation and organization of the tissues and show evidence of the interrelationships of virus and cell in the process of elaboration and release of agent from the cell. In the cases of all cell types observed, formation of virus particles occurred by the process of cell membrane budding.

A micrograph of a segment of normal glomerular corpuscle (53) is shown in figure 20 for comparison with the analogous neoplastic structures. Well-developed hilum membranes enclose the normal complement of central mass cells, and the whole is surrounded by small capillaries showing endothelial and red blood cells. External to the vascular system are the visceral epithelial cells, podocytes, with the intricate arrangement of cell processes and pedicels (55) applied to the membrane wall surrounding the capillaries. The external layer of Bowman's capsule consists of thin, flat epithelial cells with commensurately thin nuclei.

In the neoplasm all the various elements of the normal nephron could be identified but were always of a low order of differentiation and organization. The glomerular corpuscle of figure 20 shows poorly arranged hilum membranes and only vestigial central mass cells. Podocytes are present but are without the pedicel system, and no capillaries are seen. Cells of the parietal layer of Bowman's capsule are cuboidal and otherwise poorly differentiated. Bowman's capsular space usually contained much virus liberated in all probability, as will be seen, by the primitive podocytes. Poor differentiation and organization are evident also in the tubular structures in the same micrograph.

Capillaries containing red blood cells were seen frequently in association with masses of podocytes of primitive morphology in poorly differentiated glomerular corpuscles. This finding, illustrated in figure 21, indicates the presence and functioning, even in these very primitive glomerular tufts, of erythropoietic cells observed in the corresponding normal structure.

In general, somewhat higher levels of differentiation and organization were attained by the tubular structures (fig. 22) than by the more complex glomerular corpuscles. Nevertheless, the cells exhibit marked deviations from the normal.

Podocytes of low order of differentiation appeared to be particularly prolific in the elaboration of virus as might be judged by the large number of virus particles frequently associated with these elements. Evidence of formation of particles by budding is seen in figures 23 and 24. The process does not differ in principle from that of elaboration of virus by cells of chicken (8, 9, 12, 13) and mammalian (61-63) virus-induced tumors.

Of much interest are cytoplasmic aggregates of material sometimes seen in the podocytes. The masses (shown in figures 23, 24, and 25) are not enclosed but merge with the cytoplasm. Such aggregates, seen first in Rous sarcoma cells (2, 5) and designated as viroplasm, have been observed in neoplastic cells in birds diseased with other strains of avian tumor viruses. The aggregates consisted principally of electron dense granules of the size of ribosomes, which in these cells have been designated as virosomes (26). Interspersed among the granules (fig. 25) were indefinite structures with the appearance of poorly formed spheres--virospheres. Occasionally such viroplasm was close to the budding cell membrane (fig. 24), but there was no regularity of association of viroplasm with budding. The significance of the viroplasm has not been determined. There is some basis for the belief, however, that the material may represent precursor of virus (2, 26) and the result of abortive processes of virus elaboration in the cell cytoplasm, where all the components necessary for synthesis of the agent are not available.

An infrequent phenomenon apparently concerned with virus formation was observed in cells of distal convoluted tubules. Portions of two cells from a distal convoluted tubule of a normal avian kidney illustrate (fig. 26) the canalicular loops (55) formed by extensions of the cell membrane into the cell substance. Corresponding cells of the nephroblastoma (fig. 27) also show canaliculi in small numbers and of poor distribution. Collections of virus particles lie in walled or vacuole-like structures. Close inspection reveals the probability that the virus particles are situated in expanded lengths of canaliculi, in which the virus may have been formed by budding of the canalicular wall. This interpretation seems the more acceptable because the canaliculi are derivatives of the cell wall with the potentialities of virus budding.

Frank sarcomas occurring in the nephroblastomas were of the spindle-cell variety (fig. 28). The cells were usually very active in the formation of collagen. As in the Rous sarcoma (13, 14), virus was elaborated by these cells by budding (fig. 29). Buds and virus particles were present in small numbers in these sarcomas, but they were of typical appearance.

Cartilage was found on gross examination in only a small proportion of the growths. The characteristics of the chondrocytes within the substances of the cartilage are seen in figure 30. Evidence of division of the chondrocytes is indicated by the clam-shaped cells having excentrically located nuclei and the clear areas between them.

An outstanding feature of the cartilage was the arrangement of virus particles about the cartilage cells. As in the case of the podocytes, the chondrocytes seemed very active in formation of virus particles by budding, and buds were seen with great frequency in the cell membrane. The evidence of the continuing activity of the cells to elaborate virus was recorded by the particles trapped in the cartilage matrix.

It was evident that the chondrocytes were derived through progressive stages of differentiation of the stromal cells of the character of incompletely differentiated mesenchyme. (See fig. 35). Transitional cells giving rise to the chondrocytes, shown in figure 31, exhibit various intensive activities. In figure 31, the cytoplasm of the upper cells has already differentiated to resemble that of chondrocytes. At one side, one of the cells displays great activity both in the formation of collagen fibers and budding of virus particles at the cytoplasmic membrane. On the opposite side of the same cell adjacent to the cartilage, cartilage fibers are separating from the membrane from which virus particles are likewise budding. Fully differentiated chondrocytes show no evidence of collagen formation but are active in the elaboration of cartilage fibers and virus.

An interesting phenomenon was sometimes seen in association with virus budding from cells highly active in collagen elaboration. In the meshes of collagen fibers (fig. 32), there are spheroidal bodies of wrinkled external outlines. Structures are in evidence in an intermediate position between wrinkled bodies having clear centers and typical virus particles with central nucleoids. The nature of the spheroids has not been established, but it seems possible they may represent aberrant virus particles with collagen-related material introduced into the particle membrane in the process of budding simultaneous to the elaboration of collagen at the cell membrane.

An analogous phenomenon was apparent in association with virus budding and formation of cartilage fibrils by the chondrocytes. The elaboration of large amounts of virus by the chondrocytes is illustrated in figure 33, which shows the mixture of virus and cartilage fibrils about one of these cells. In the higher magnification of figure 34, the simultaneous formation of buds and the splitting of cartilage fibrils from the cell surface are evident. There are shown also numerous virus particles, many of which contain internal striae bearing a strong resemblance to segments of cartilage fibrils. Judging from the morphologic evidence, it would appear that the structures inside the virus particles may, indeed, represent pieces of cartilage fibrils incorporated in the particles budding from the cell membrane active at the same time in elaborating cartilage fibrils.

The ultrastructural aspects of the stromal cells always associated with the various other tissues of the nephroblastoma (figs. 11 and 12) are illustrated in figure 35. Such cells were of indeterminate character, exhibiting some elements of morphology similar to that of fibroblasts and others to those of epithelial cells. In these respects, the cells of a large proportion of the stroma resembled many of the epithelioid elements of the nephrogenic buds in the normal postembryonic chick kidney (fig. 4). Such cells were of ubiquitous distribution in all tumors, and a constant finding was the presence of all grades of elements of morphology

transitional between the stromal cells and all other cell types. The stromal cells were obviously the precursors of chondrocytes (fig. 18) and of cells differentiating to form osteoid. Microscopic areas of keratinizing epithelial cells occurred as isolated islands in sheets of stromal cells, and formations of spindle-cell sarcoma merged indistinctly with the undifferentiated stroma. There was no direct evidence of the origin of the relatively highly differentiated and organized nephronic structures from the stroma. It appears significant, however, that the purely epithelial structures of glomerular corpuscles in various states of differentiation sometimes occupied widely separated areas of large tumors. This suggested the possibility that the stromal cells may also have constituted the precursors of the neoplastic elements.

The ultrastructural aspects of the initial stages of normal nephron development are shown in the micrograph (fig. 36) of the cortex of the metanephros of a 13-day chick embryo. An ingrowing collecting tubule bud has pushed into the nephroblastema close to the cortical boundary of the kidney. About one horn of the bud there is the small inverted comma-shaped mass of epithelial cells in a very early stage of differentiation to the epithelium of the nephron. On all sides of the collecting tubule and of the precursor of the nephron are seen the undifferentiated mesenchyme-like cells of the nephroblastema. In the region of the tail of the comma, there are elements close to the tubule but which exhibit morphology transitional between nephroblastema and epithelium and bear a close resemblance to the undifferentiated stromal cells of the tumor (fig. 35).

Discussion

In the present discussion there have been considered certain aspects of two major neoplastic manifestations of host response to the BAI strain A virus. Myeloblastosis and nephroblastoma, however, represent only a part of the total spectrum of response to the same agent. In addition, there also occur lymphomatosis, osteopetrosis, sarcoma, nonneoplastic changes in the thymus (64, 65) and in the pancreas, and probably other conditions as well. It is notable that thus far no evidence has been seen of the induction of the second leukemia, erythroblastosis, by this agent.

Investigations in the past half century have shown that the most outstanding biologic phenomenon of the virus-induced avian tumors is variation in both quantitative and qualitative response of the host. It was not until recently that systematic studies under controlled conditions clearly revealed the nature of factors exerting major influences on the character of response. It is now quite evident (16) that the kind of tumors and the incidence of each in test populations of chickens exposed to some of the well-studied strains of avian tumor viruses are dependent on dose of virus, age and genetic constituion of the host, and obviously on the care and duration of observation of the birds under study. With proper consideration of the various factors, the spectrum of response obtainable with certain strains is reproducible with considerable fidelity, and, to a large extent, the spectrum is closely related to the virus strain. Such findings justify the conclusion that, although the different strains are biologically related, each differs from the other in that attribute which fosters initiation of cell-virus interaction.

Despite the evident differences between the strains, there are observed certain similarities in the characters of host response. Lymphomatosis, for example, is a response common to infections with Rous sarcoma (66), RPL 12 (67), BAI A (23), and R (23) strains, and other conditions are likewise common to two or more of the strains. In principle, the disease lymphomatosis induced by any one strain is the same as the condition resulting from the other strains. It is thus evident that specific cell response, if it occurs, is a manifestation of cell potentialities for neoplastic behavior and not the result of specificity of virus attributes.

Study of the neoplasms and nonneoplastic diseases induced by the BAI strain A virus, particularly myeloblastosis and nephroblastoma described here, provides an excellent illustration of the contribution host potentialities can make to the characters of disease and to the intimate aspects of cell-virus interaction. Manifestation of host-cell potentialities is particularly well demonstrated by the complex tissue composition of the nephroblastoma and the principles of growth and differentiation governing the origin of the various cell types. In the matter of cell-virus relationships, it is evident that the mechanisms of virus synthesis and final elaboration and release are functions characteristic of the cell and not of the virus.

Response of the myeloid hematopoietic tissue in myeloblastosis exhibits notable features of cell specificity, which for the most part is concerned with virus elaboration. Morphologically, the myeloblasts in the blood of leukemic chicks are indistinguishable from the corresponding primitive cell in normal bone marrow. As already mentioned, unequivocal evidence of virus associated with the diseased cells (17, 19) is infrequently observed until the myeloblasts are cultured under appropriate conditions *in vitro*. Response is then immediately apparent in the appearance of inclusion-like bodies or viroplasts. Viroplasts are specific to the myeloblasts in the sense that bodies like them have not been unequivocally identified in the neoplastic cells of other avian tumors induced by viruses.

Strong presumptive evidence suggests that this unique aspect of myeloblast response is related to the potential of the cell in the formation of granules on maturation. Precursors of granules are not present in other cell types, and it seems likely that such precursor material may be the cell constituent or substance involved in the cytoplasmic integration of virus-determinative material occurring on infection of the cell. In this process there is no evidence of transformation of the cell from one type to another. Instead, the cell retains the property of continued growth exhibited by the primitive element in the bone marrow, and, in addition, the processes leading to the appearance of granules and other characteristics of maturation are completely inhibited. In all of these respects, it is clear that these features of response are related to cellular properties and not to a specific effect of the virus to divert the cell to processes beyond those intrinsic in the element.

Evidence of the operation of potentials inherent in the normal cell in the determination of the form of neoplasia is a major feature of the nephroblastoma. On the assumption that growth is derived from post-embryonic nephrogenic cell rests, it would appear that the tumor arises as the result of two separate influences--one of intrinsic origin in the normal kidney, and the other of extrinsic origin exerted by the viral agent.

In all probability, the nephrogenic buds represent collections of primitively differentiated nephroblastemic cells, which, for unknown reasons, were unable to complete the process of nephron formation. This may have occurred as the result of faulty action of normal inductors (58) or possibly failure of the primitive nephron to effect junction with the collecting tubule. Under any circumstances, it may be assumed that the transition in differentiation from nephroblastema to the nephrogenic tissue was due to the initial influence of the normal inductors.

Infection by the BAI strain A virus results in resumption of growth of the nephrogenic cells and the initiation of a series of processes of differentiation to give rise to a variety of neoplasms of widely different cell types. One of these always present in the nephroblastoma consists of epithelial structures closely resembling the normal nephron. Although there are great variations from one tumor to another and within the same growths, it is plain that the nephrogenic cells under the stimulus of the virus are capable of differentiation to every cell type and structure of the normal nephron. These include all the cellular elements of the glomerular corpuscle and the total tubular system. It is particularly notable that capillaries and red blood cells occur in the neoplastic glomerular tuft just as in the embryonic development of the corresponding structure of the normal nephron. This finding signifies that the neoplastic derivatives of the nephrogenic cells are differentiated to the levels of erythropoietic tissue in the glomerular tuft.

From these findings, it may be judged that the epithelial components of the nephroblastoma represent the expression of the inherent capacities of a single cell type for progressive differentiation to yield the complex structure of the nephron. It is notable that in these processes differentiation passes through the stages of mesenchyme (nephroblastema) to epithelium (primitive nephronic anlage as shown in figure 4, and again to mesenchymal elements (capillary endothelium and erythropoietic tissue). Furthermore, growth of every epithelial component is not continuous. Instead, growth of the individual cell types of the nephronic segments ceases with the attainment of the terminal stage of differentiation. In all of these respects, the neoplastic nephronic elements differ from the normal only in aberration of cell differentiation and in lack of refinement of organization of the various structures. It is thus clear that the action of the virus induces no new cell type. On the contrary, the effect of the virus on the cell is entirely analogous to that of the normal renal inductors.

The inherent potentialities of nephrogenic cells, either at the level of nephroblastema or at the postembryonic renal rests, to differentiate to other nephroblastoma tissues is not as clear as that pertaining to development of aberrant nephronic structures. The stromal cells, constituting the ubiquitous structure of all nephroblastomas were clearly derived from the nephrogenic cells. In turn it was certain that cartilage, osteoid, and spindle-cell sarcoma originated by processes of differentiation of the stromal cells. It was of importance to note that the transition from stroma to these components was not of the character of sudden transformation. On the contrary, all morphologic forms of transition were seen.

As the findings showed, expression of cell influence on the infectious process was apparent not only in the manifestation of the pathologic response but also in the differences in the mechanisms of virus synthesis and the character of the virus particles elaborated. Formation of the agent in the myeloblast seems to occur in special cytoplasmic inclusion bodies, and it has been presumed that the particles are expelled from vacuolated viroplasts without involvement with the cell membrane. It has been the hypothesis, further, that the viroplasts constitute virus-induced derivatives of the precursors of the granules of the myeloid series of cells. Under these conditions, then, the peculiar occurrence of viroplasts and the mechanism of virus synthesis are obviously related to cell potentialities and not to special directive influences of the virus itself.

The critical importance of cell properties on the final elaboration of virus is further emphasized by the mechanisms apparent in the renal tumor. In this neoplasm, virus is assembled and released from the various cell types by budding. Presumably both myeloblastosis and the renal tumor were induced by the same virus. Consequently, it is clear that the exact mechanism of virus production was not related to specific influence of the virus but by capacity of cell response. Another difference related to cell type was the seeming intensity of the budding process as judged by the number of buds associated with the cell surface.

Finally, it could be seen that the character of the virus particles was alterable by complications related to cell membrane activities at the locus of virus synthesis. This was particularly well shown by the character of virus particles that budded by cartilage cells active in the release of cartilage fibrils at the cell membrane. Under these conditions the micrographs showed structures in the virus particles that resembled fragments of cartilage fibrils suggesting inclusion of the material incidental to assembly of the external covering of the virus particle by incorporation of cell membrane. An analogous complication of particle formation was also suggested by the wrinkled spheres associated with cells highly active in the elaboration of collagen and virus buds simultaneously.

The findings with myeloblastosis and the renal tumor serve the purpose of disclosing the characteristics and varieties of cell response to a single avian tumor virus. Of greater significance, however, are questions relative to the principles of biologic activity responsible for the processes observed. Lack of knowledge of the exact nature of virus-cell relationships responsible for the initiation of cell behavior characteristic of neoplasia and virus synthesis leaves freedom for the most varied speculation.

The theory possibly more prevalent than others at the moment envisions transformation of normal cells to neoplastic cells by alterations in genetic constitution (45, 68). It is further conceived that mutation is effected by integration of viral determinative material with that of the cell. Although there is no basis for denial of the possibility, there is equally no reason, except remote analogies with bacteriophage host relationships, to regard the hypothesis as more than a most superficial guess. There is much reason to doubt that induction of neoplasia by the BAI strain A virus is effected by such mechanisms. Presumable genetic

interaction of cell and virus implies specificity of the resultant with respect to both cell change and virus elaboration, and further that cell transformation might be of the order of a sudden change. In none of these respects is there evidence of specificity either in the type of cell infected or in the character of the neoplasm. The terminal stages of the kidney tumor are obviously reached by a series of both continuous and discontinuous processes of cell differentiation. Progression of nephrogenic cells to the abnormal nephronic structures passes through the same stages as development of the normal nephron. Cartilage, spindle-cell sarcoma, and osteoid are also derivatives of progressive differentiation of nephrogenic cells. Lack of specificity is notable also in complications of virus formation.

In the end it would appear that, in the instance of the kidney tumor at least, the action of the virus resembles closely that of the normal renal inductors. Indeed, in the entire spectrum of response to the BAI strain A virus, it would appear that the virus behaves with many of the attributes of an inducer which stimulates the cell to the manifestation of one or more processes of differentiation in accord with potentialities inherent in the element affected.

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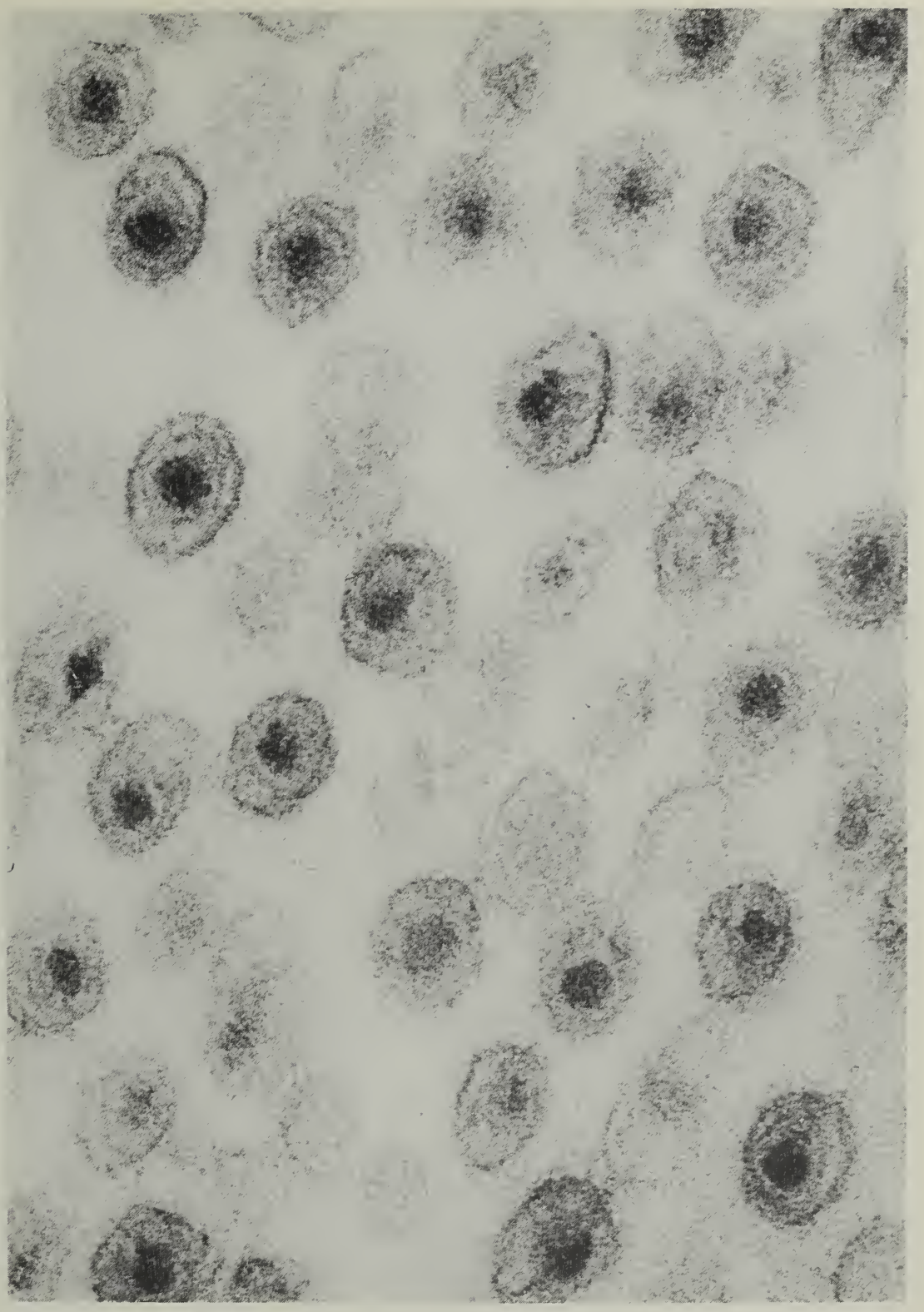

Figure 1.--Electron micrograph of thin section of BAI strain A virus purified from the blood plasma of a chicken with myeloblastic leukemia. X 160,000.

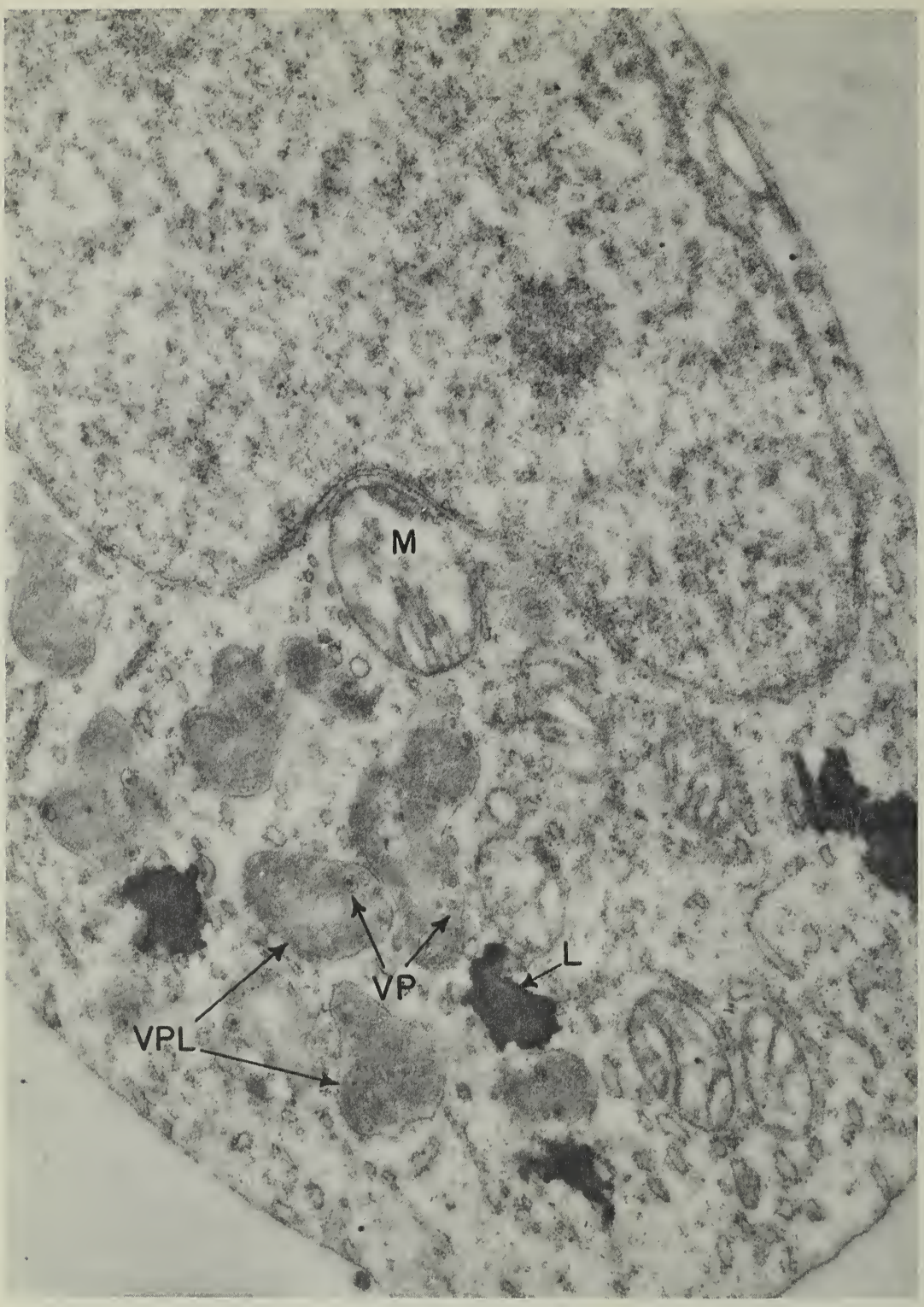

Figure 2.--Myeloblast from a 2-day old culture of cells taken from the circulating blood of a bird with myeloblastosis induced by the BAI strain A virus. Note a large number of viroplasts (VPL) containing few virus particles (VP). (M) = mitochondria and (L) = lipid body. X 29,000. (17).

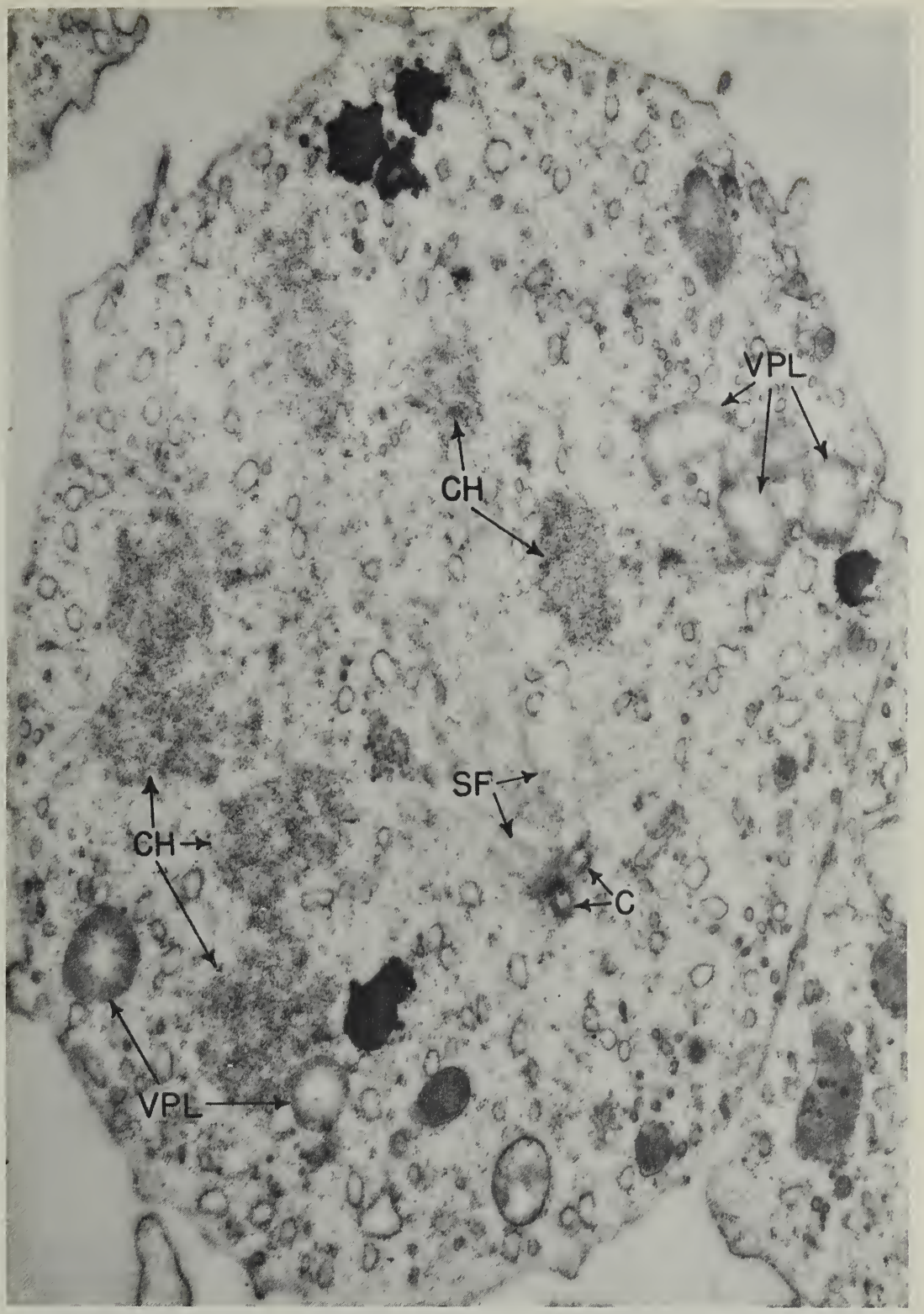

Figure 3.--Mitosis of myeloblast in tissue culture. Spindle fibers (SF) extend from centrioles (C) toward chromatin (CH). Viroplasts (VPL) are distributed at opposite poles of dividing cell. X 19,000. (19).

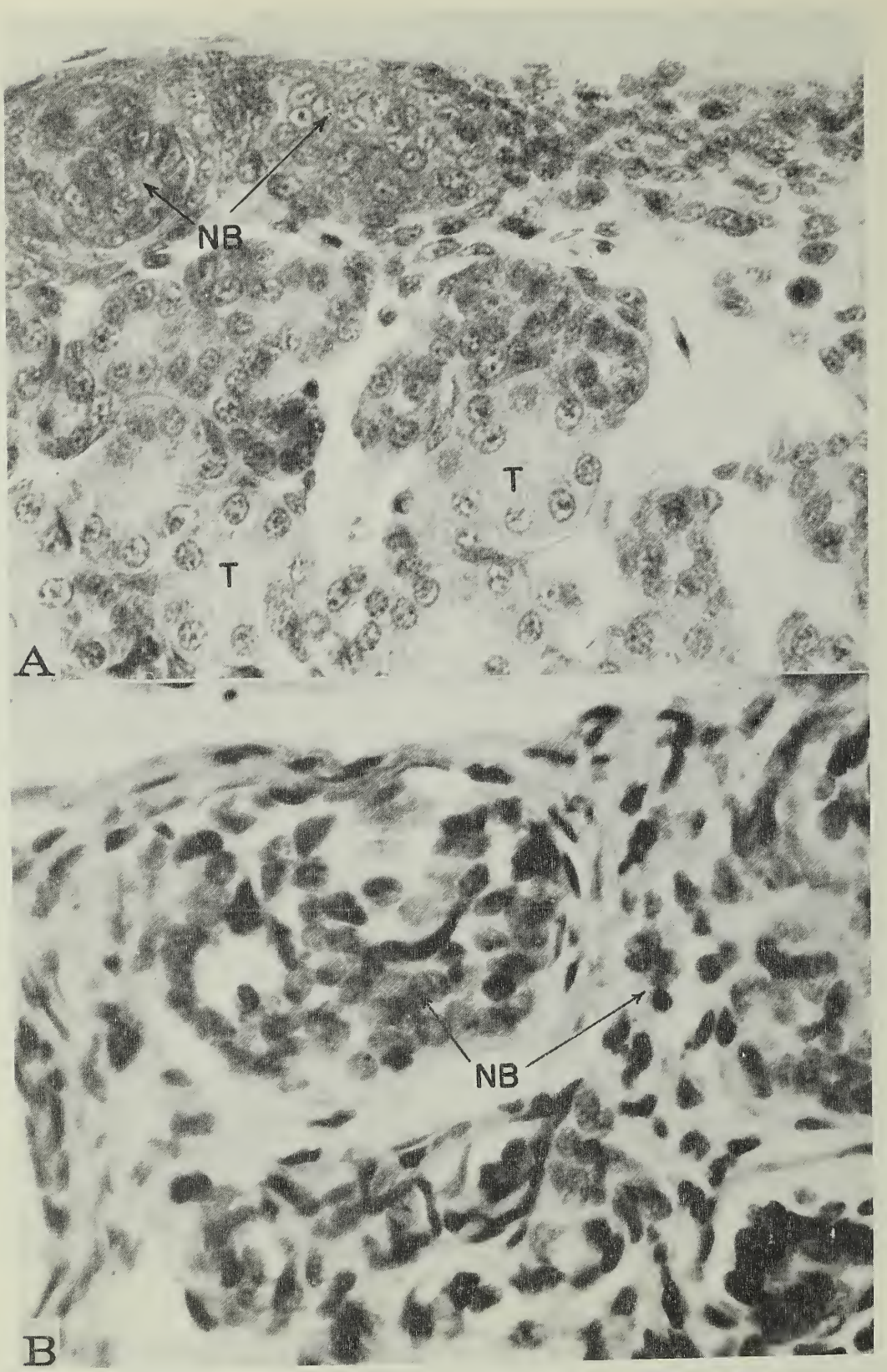

Figure 4.--Nephrogenic elements (NB) residual in subcapsular buds in kidney A, of newly hatched chick surrounded by normal nephronic structures (T). B, of child 5 weeks old. X 743. (25).

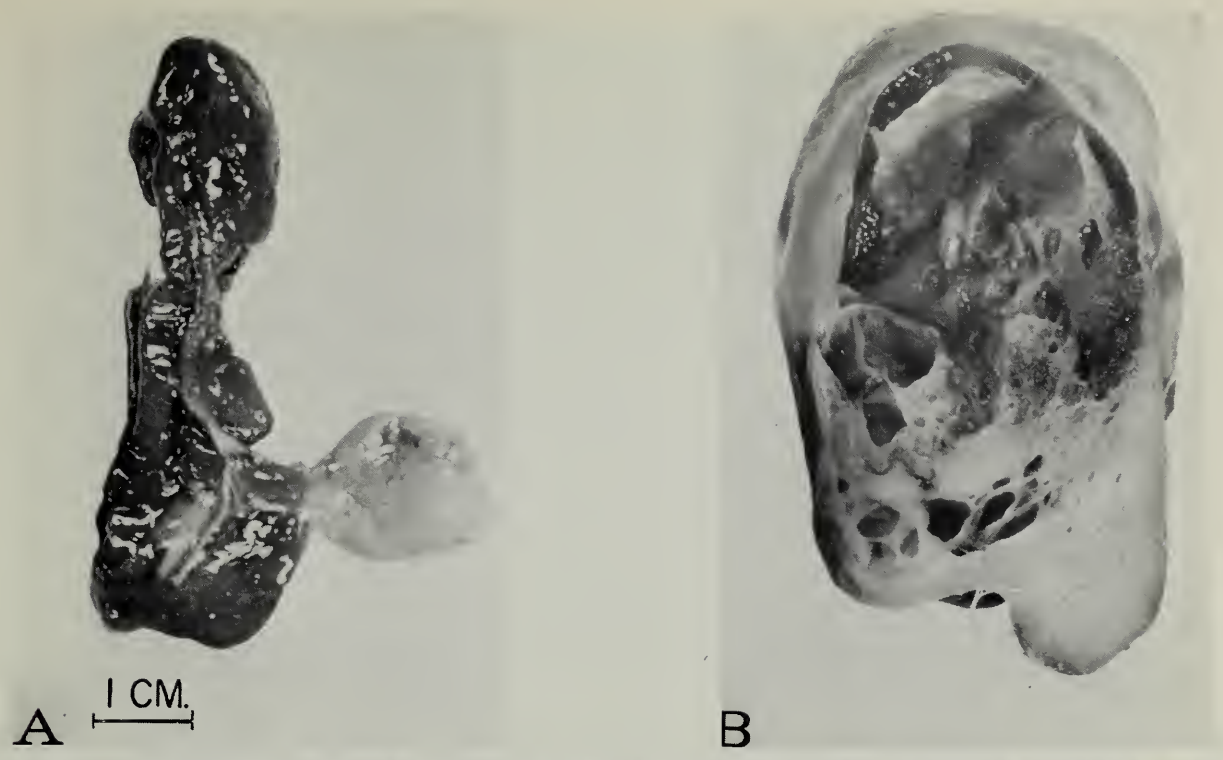

Figure 5.--A, growth in posterior pole of right kidney, which arose superficially and was attached to the renal cortex by a stalk of firm tissue. B, section through the growth shows stalk and large and small cystic expansions. (25).

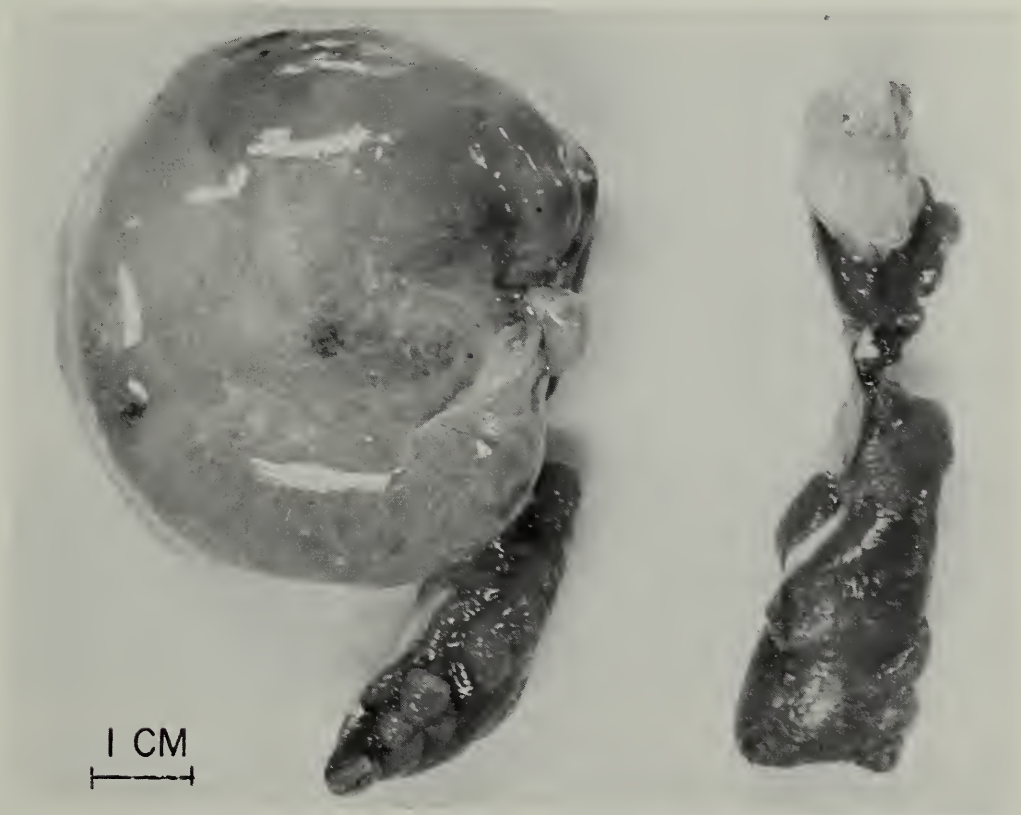

Figure 6.--Bilateral single renal growths. Smaller tumor in anterior pole of left kidney and larger growth in anterior pole of right kidney. (25).

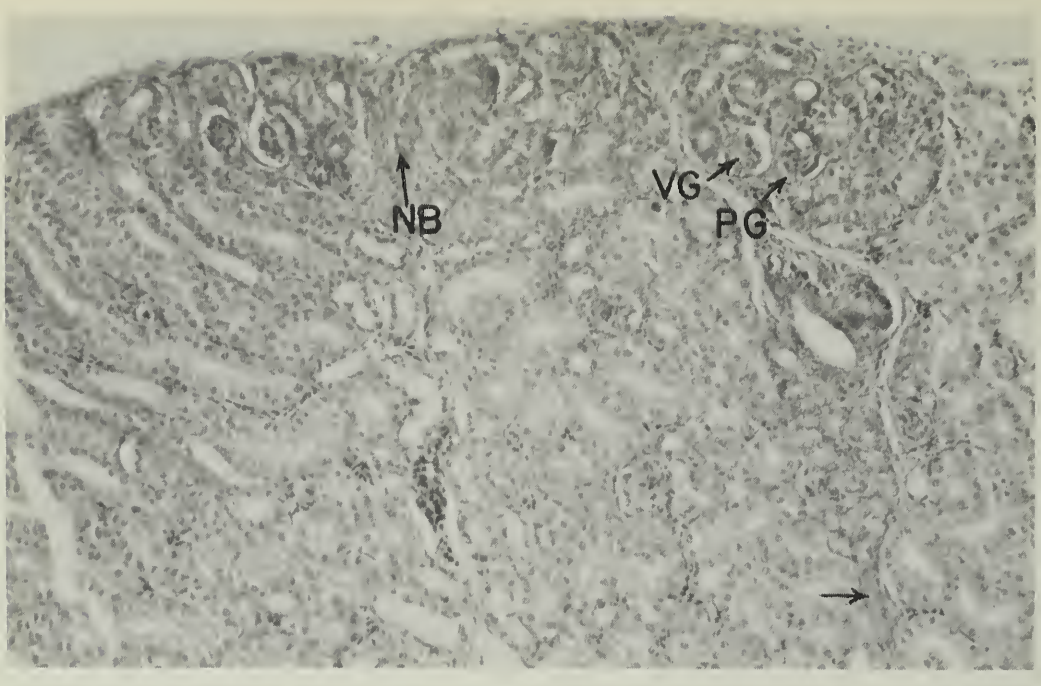

Figure 7.--Nephrogenic bud beneath renal capsule extends deeply into cortex (arrow) of kidney of normal 37-day old chick. Tubules and vestigial (VG), and primitive (PG) renal corpuscles are enclosed in sparse connective-tissue stroma. Undifferentiated nephrogenic elements (NB) are situated in nests and strands. X 204. (25).

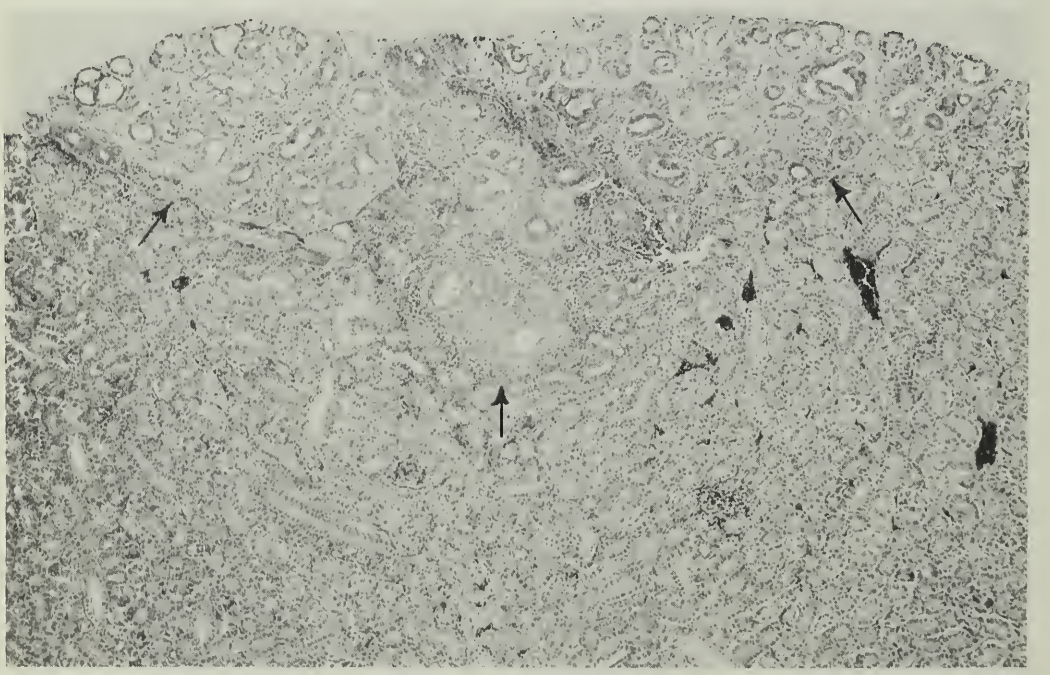

Figure 8.--Initial stages of tumor growth of tubules and primitive glomerular structures lobulated by relatively heavy stroma of unorganized, incompletely differentiated mesenchymal cells. See UM in fig. 12. Abnormal growth (arrows) joins normal cortical structures without encapsulation. X 79. (25).

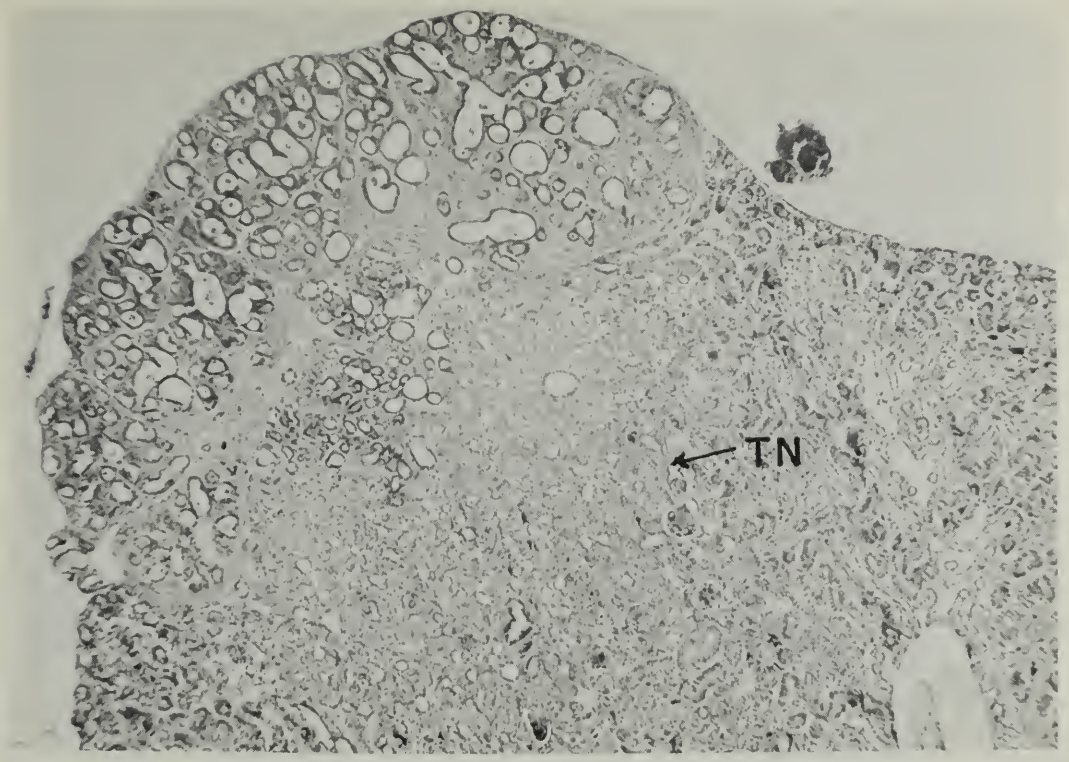

Figure 9.--Early progressive growth of unorganized epithelial and mesenchymal elements extend into normal cortex. Boundary between normal and tumor tissue (TN) is sharp, but growth is not encapsulated. X 57. (25).

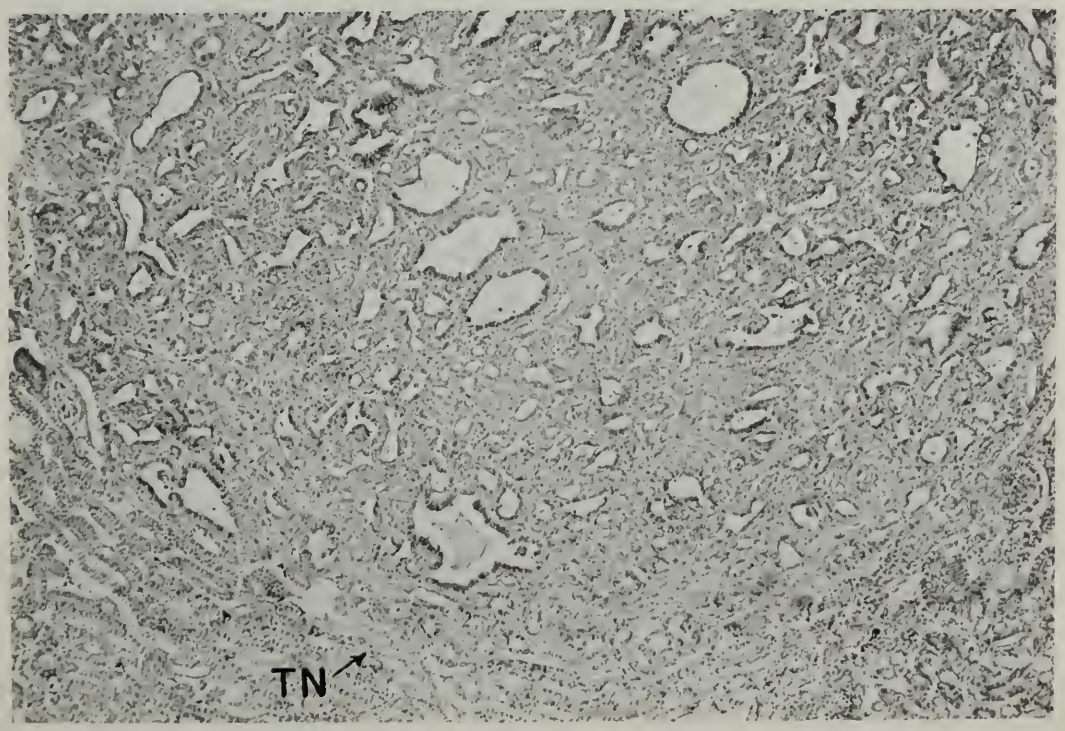

Figure 10.--Another large tumor arising in kidney structure similar to that shown in figure 9. Appearance of cystadenofibrosarcoma merges smoothly (TN) with renal cortex. X 84. (25).

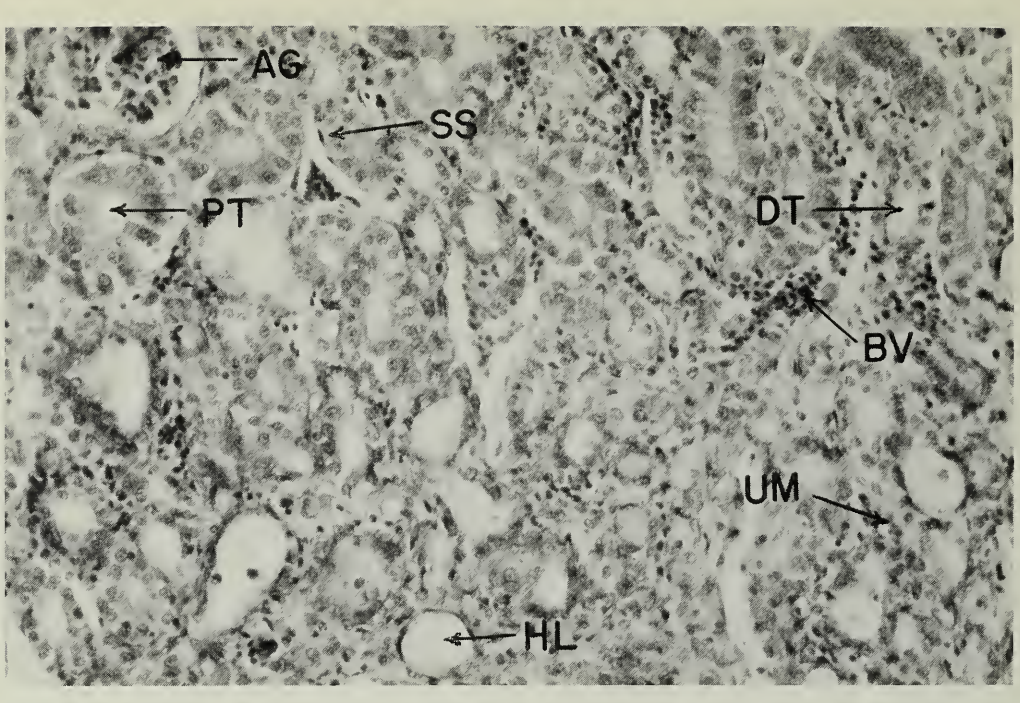

Figure 11.--Epithelial nephronic differentiation with formation of abnormal glomerulus (AG), proximal (PT) and distal (DT) convoluted tubules, and tubules of thin loop of Henle (HL). Well-vascularized (BV), delicate, normal-appearing spindle-cell stroma (SS) supports tubules. Intertubular spaces are infiltrated by undifferentiated mesenchymal cells (UM). X 288. (25).

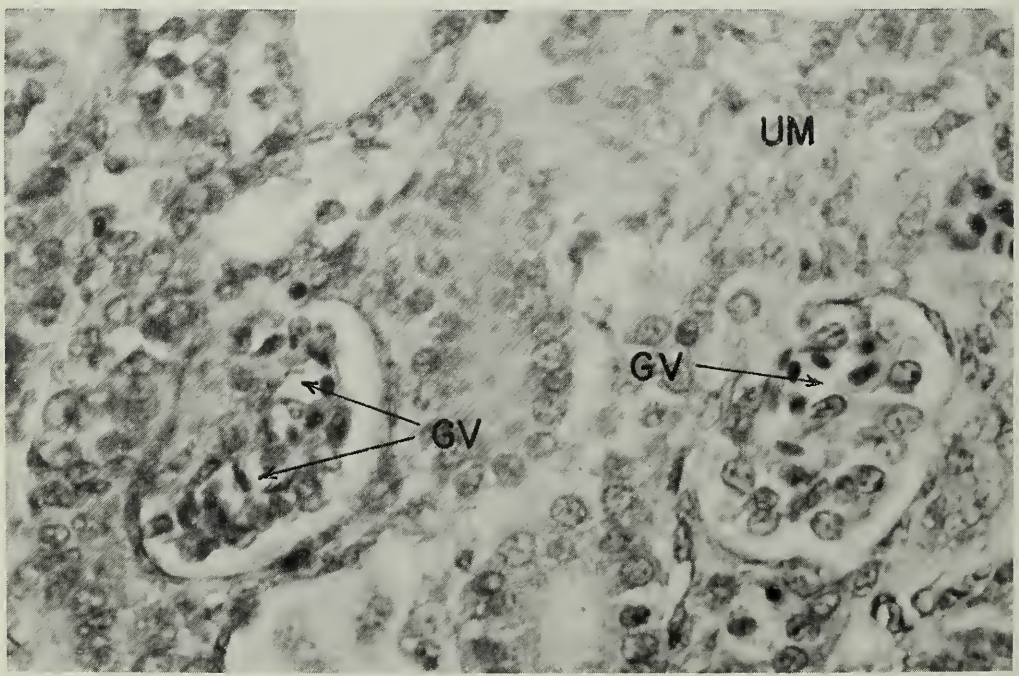

Figure 12.--Higher magnification of another part of growth shown in figure 11, which illustrates 2 renal corpuscles that closely resemble normal structures, with development of glomerular blood vessels (GV) lying in an infiltrating stroma of undifferentiated mesenchymal cells (UM). X 722. (25).

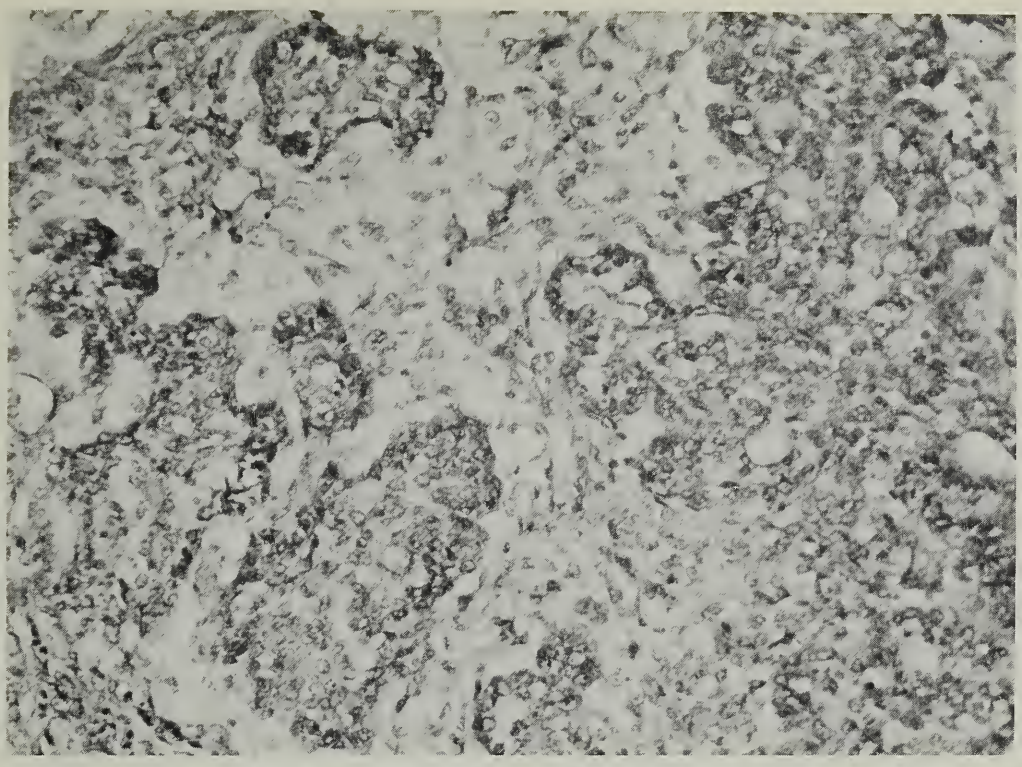

Figure 13.--Epithelial cells in pseudopapillary arrangement and scattered without organization in a merging network of fibroblastic elements. X 288. (25).

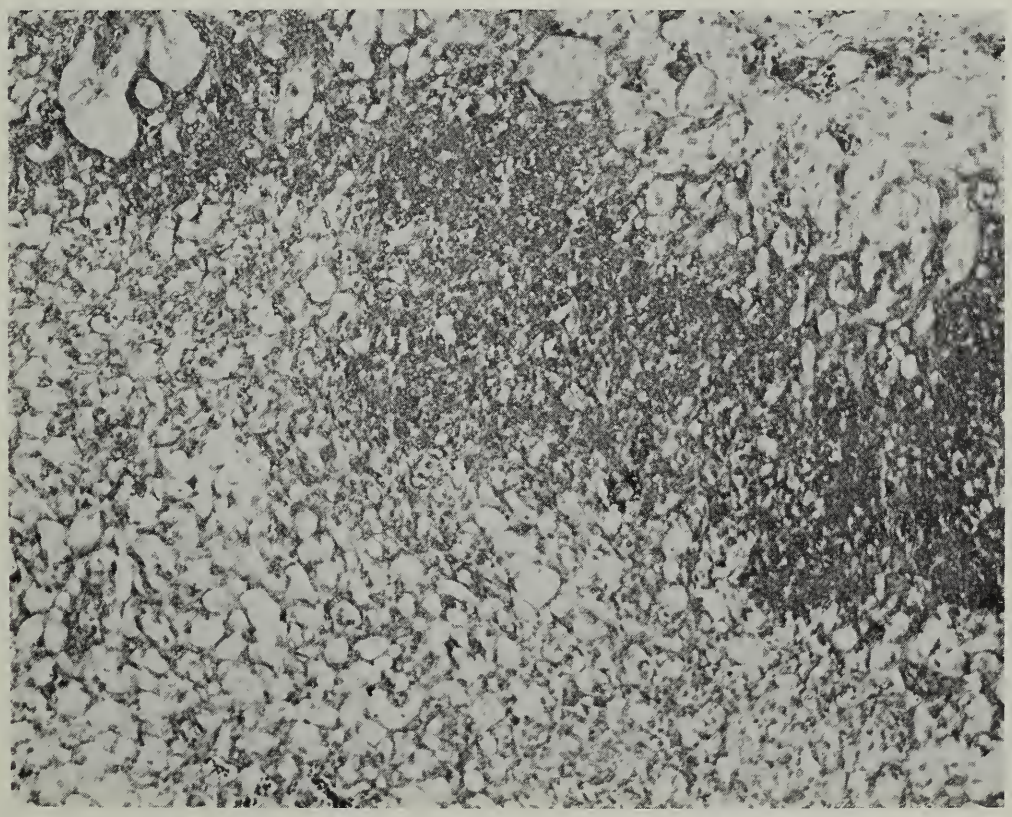

Figure 14.--Completely unorganized epithelial elements merge with mesh of fibroblasts. X 145. (25).

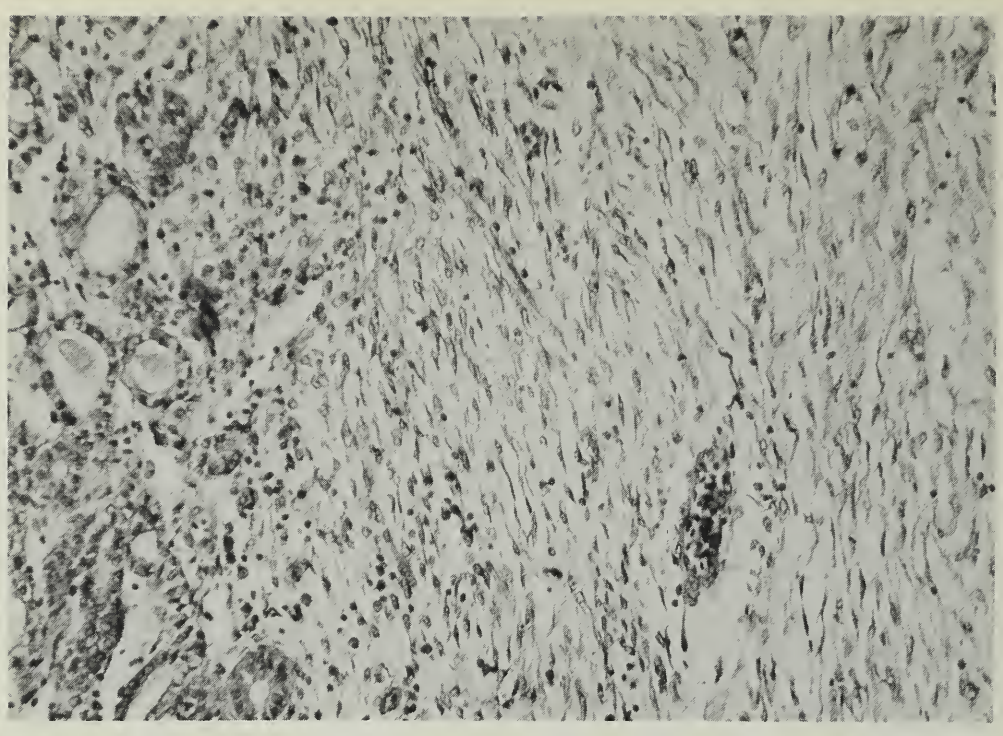

Figure 15.--Spindle-cell sarcoma with highly differentiated branching fibroblasts that occurred as a well-delineated, circumscribed growth in a tubular adenoma situated in the kidney. X 288. (25).

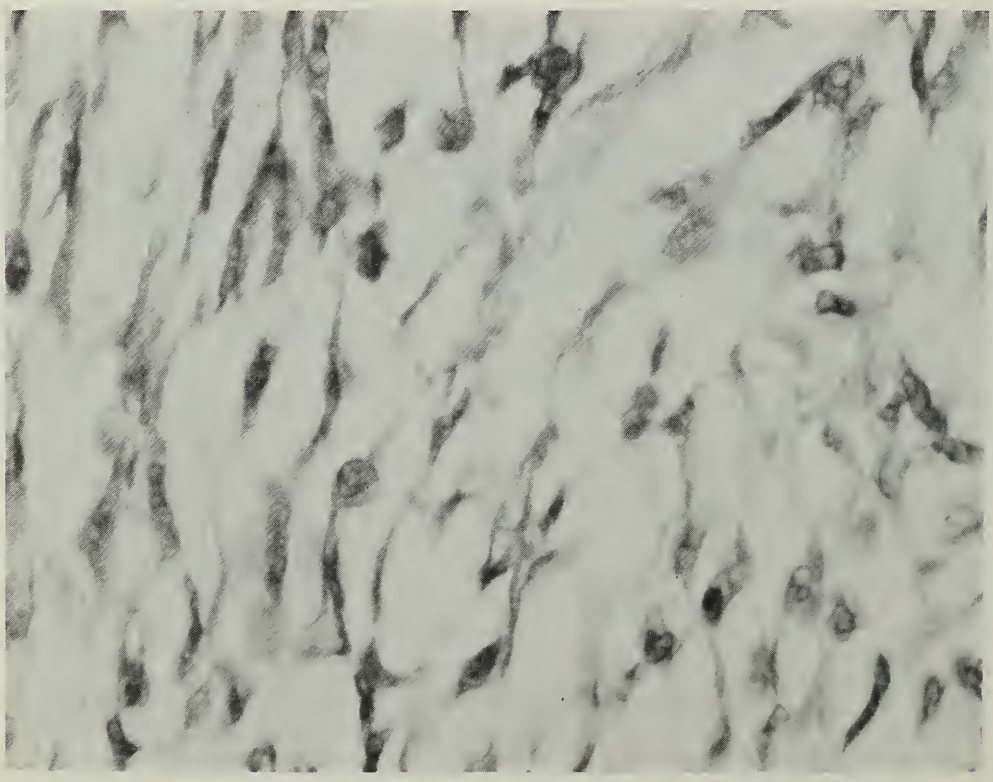

Figure 16.--Higher magnification of an area of the growth shown in figure 15, which illustrates typical orderly ramification of the cell processes of the fibroblasts. X 722. (25).

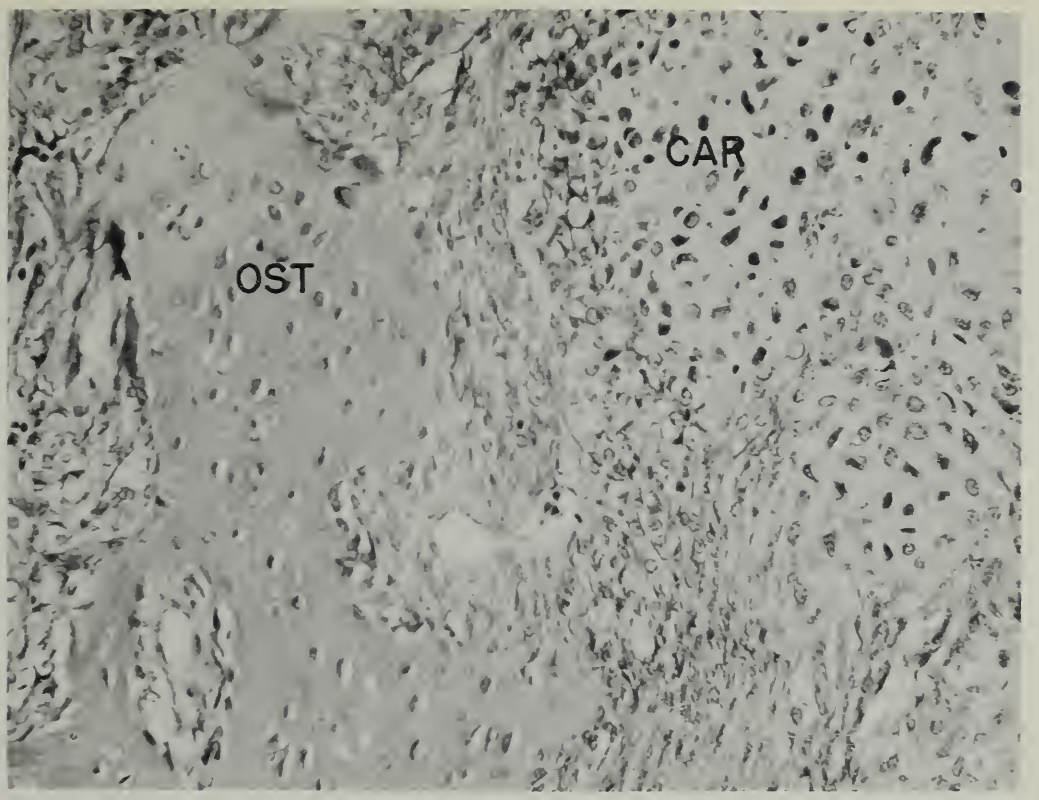

Figure 17.--Deposits of cartilage (CAR) and osteoid (OST). Chondrogenic and osteoidogenic elements are derived from mesenchymal stroma cells of varying stages of differentiation. X 288. (25).

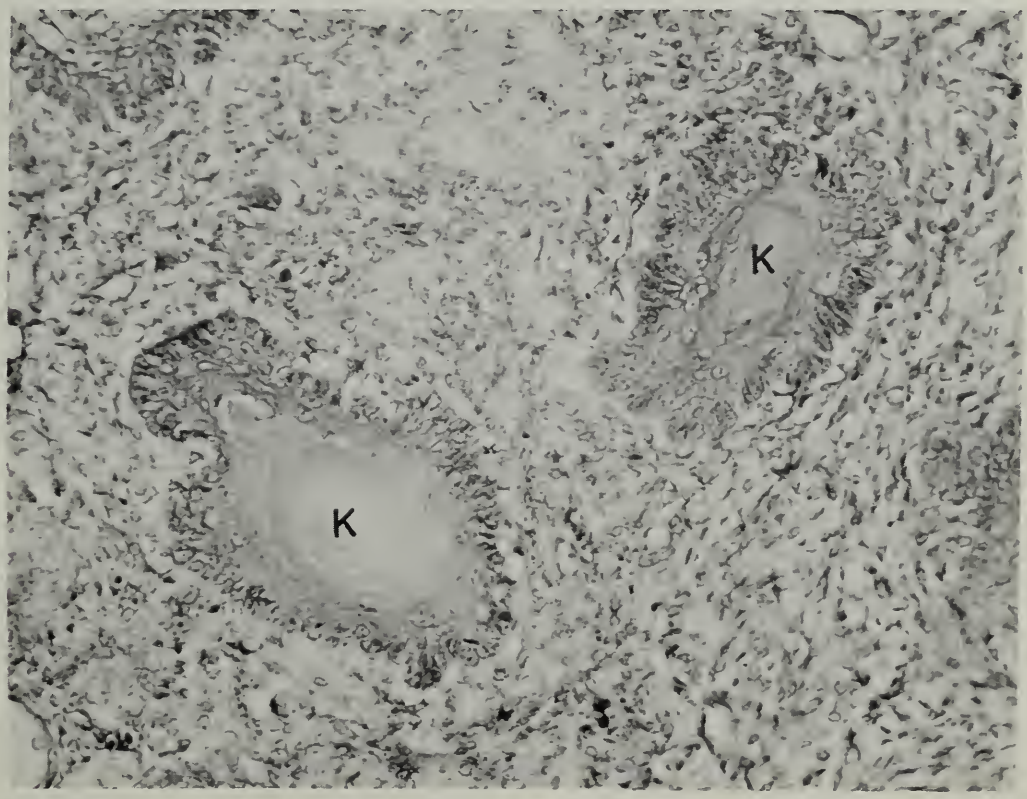

Figure 18.--Localized keratinization (K) in a stroma of predominantly sarcomatous character. X 288. (25).

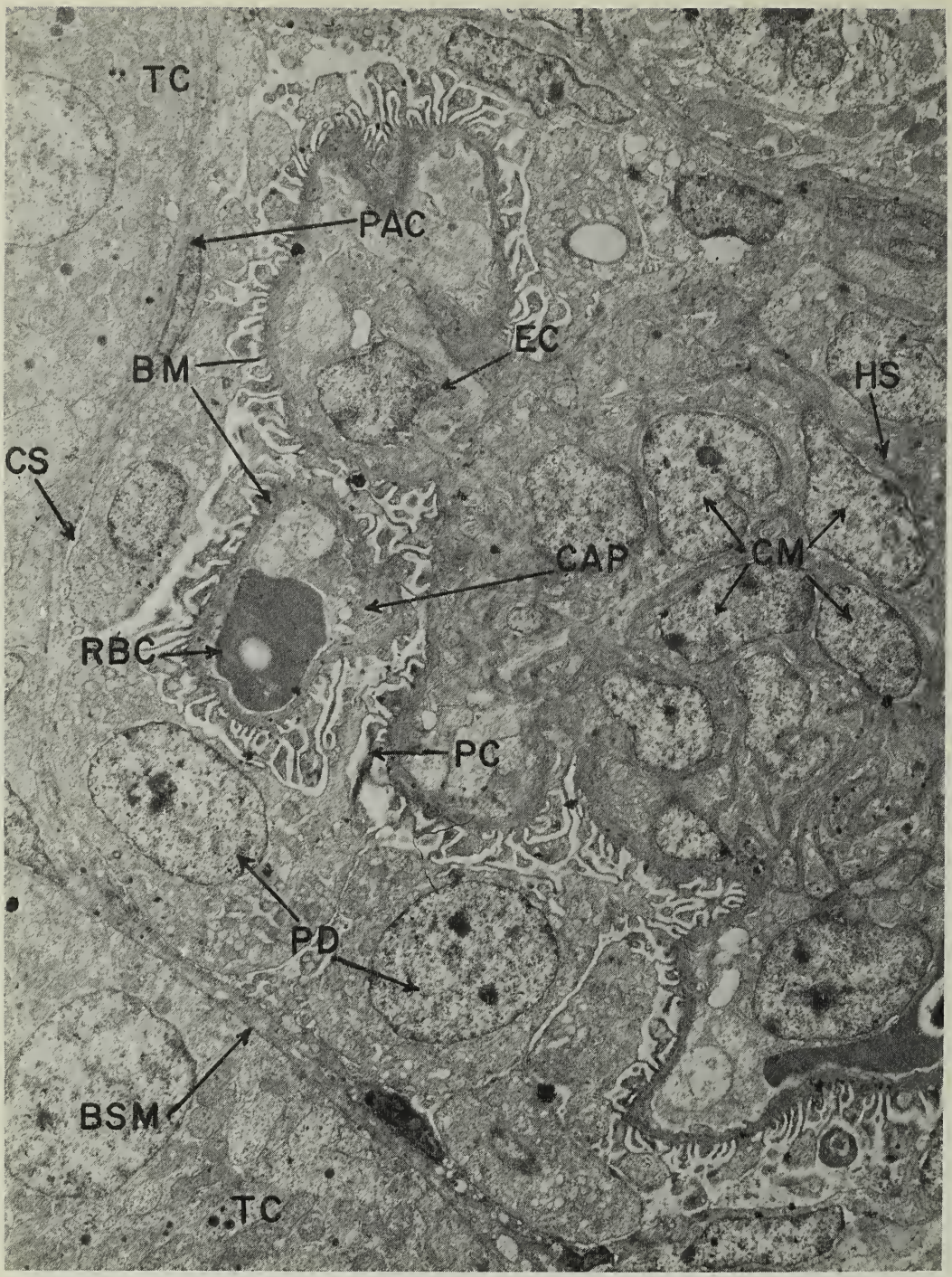

Figure 19.--Section through portion of renal corpuscle of normal avian kidney. Membranous structures (HS) that are continuous with stalk or hilum enter capsular space from the right. Epithelial cells (CM) constitute central cell mass in meshwork of stalk membranes that are continuous with basement membranes (BM) of glomerular capillary loops (CAP). Capillary loops are layered peripherally by visceral glomerular epithelial cell--podocytes (PD)--with pedicels (PC) attached to glomerular basement membrane. Glomerular capillary endothelium (EC) crowds lumen containing red blood cell (RBC). Glomerular tuft fills capsular space (CS) lined peripherally by delicate parietal capsular epithelium (PAC) with spindle-shaped nuclei. Capsular basement membrane (BSM) separates Bowman's capsule from adjacent tubular structures (TC). X 7,000. (26).

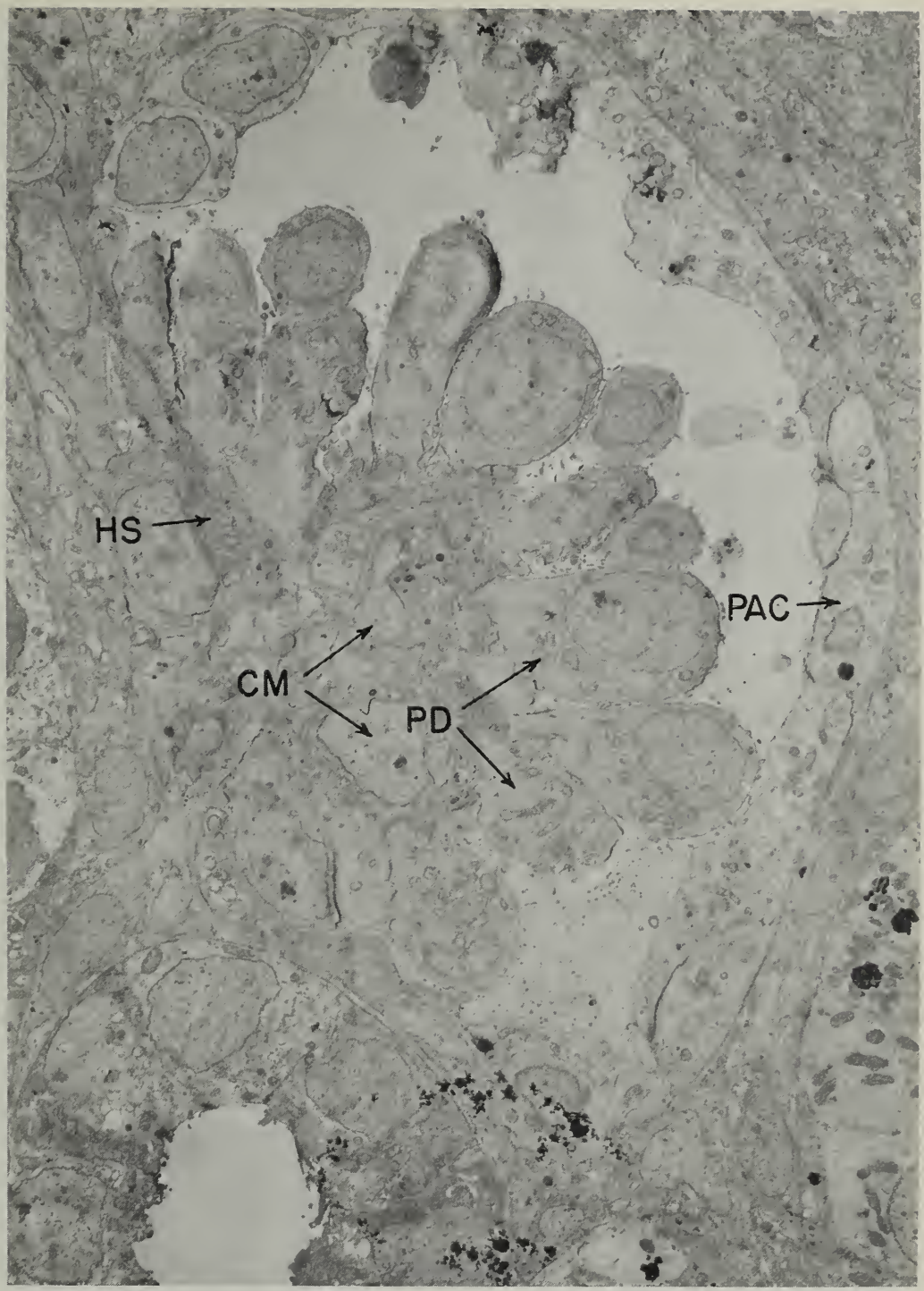

Figure 20.--Renal corpuscle of the tumor exhibits distinctive but low order cell differentiation and organization. Stalk membranes (HS) and central cell mass (CM) are prominent. Pear-shaped podocytes (PD) are applied by basal cell membranes to stalk membranes. Parietal capsular epithelium (PAC) is cuboidal. No evidence of capillary loops. X 2,700. (26).

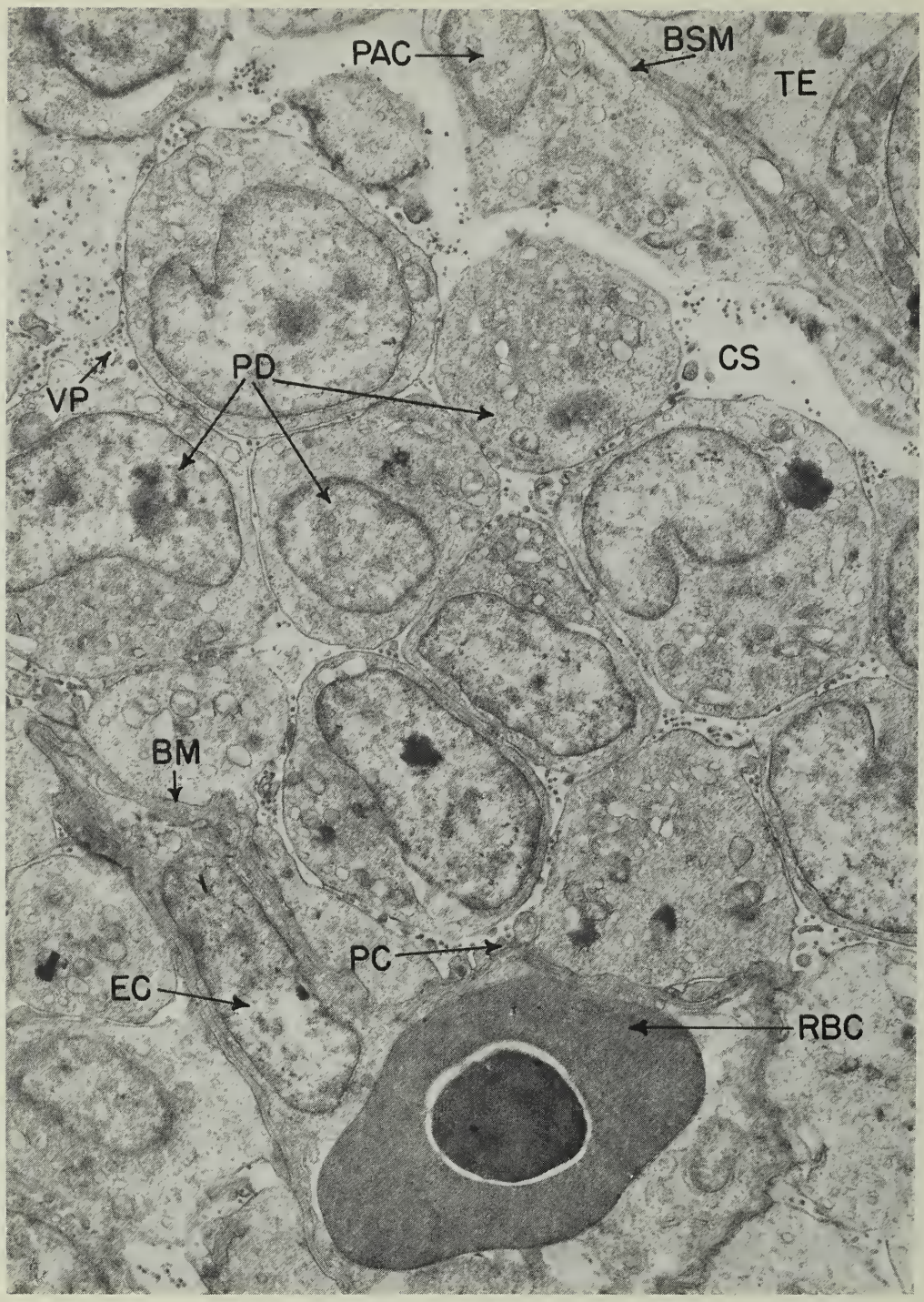

Figure 21.--Section of another glomerular corpuscle found in the nephroblastoma illustrates additional principles of differentiation and organization. The podocytes (PD) are less well differentiated than those shown in figure 20 and show few pedicel (PC) protrusions. Cells of the parietal layer of Bowman's capsule (PAC) are irregular and thick, and the capsular basement membrane (BSM) is poorly developed. A capillary is present containing an endothelial cell (EC) and a red blood cell (RBC). The capillary basement membrane (BM) approaches the normal structure. Virus particles (VP) are scattered in the intercellular and Bowman's capsular spaces (CS). Tubular epithelial cells (TE) are of low grade differentiation. X 8,000. (69).

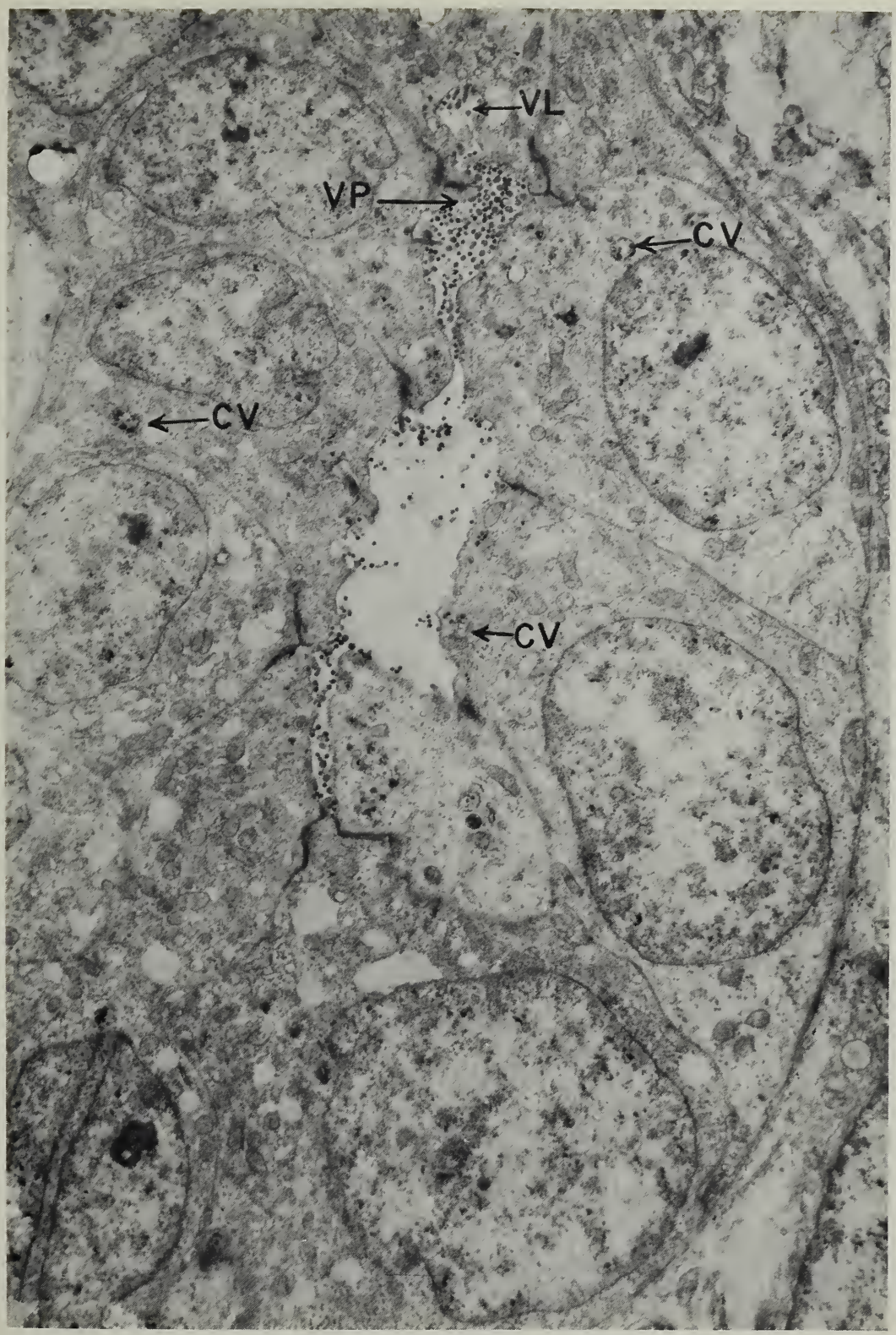

Figure 22.--Section of a distal convoluted tubule with few scattered abortive villi (VL). Cells are incompletely differentiated; mitochondria are few and abnormally distributed (figure 26). Cytoplasm is thin, and apical cell membrane is irregular. Much virus (VP) is in lumen; small collections of particles are in cytoplasmic vacuoles (CV). X 7,000. (26).

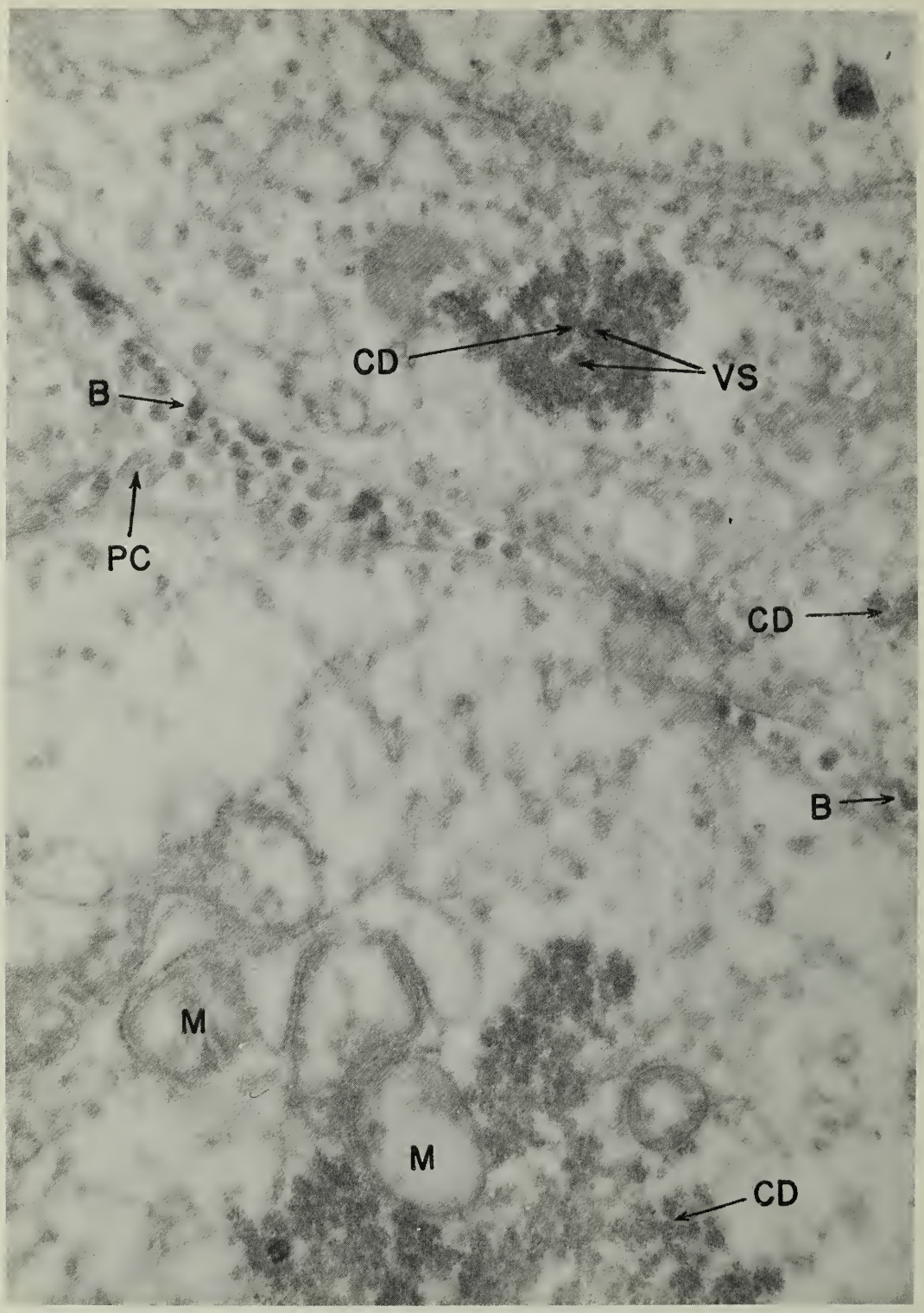

Figure 23.--Portions of two podocytes of primitive differentiation. Large and small condensations (CD) of virosomes, viroplasm, and virospheres (VS) are in cytoplasm of both cells. Virus particles are in intercellular space and several virus buds (B) are in both cell membranes. Mitochondria (M) are swollen; cristae are disrupted. Primitive pedicel processes (PC) few. X 28,000. (26).

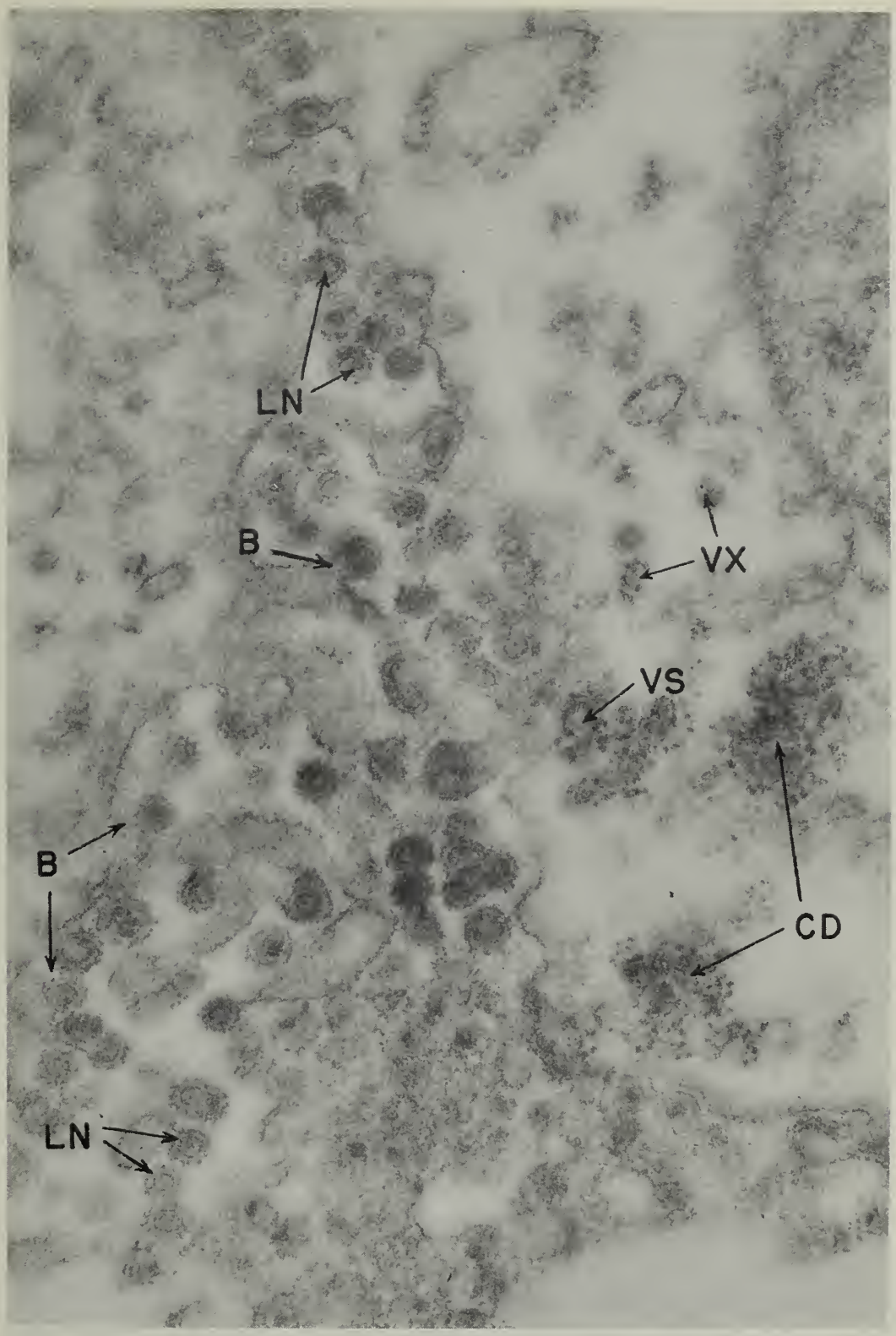

Figure 24.--Enlargement at the membranes of two cells near area shown in figure 23. Intensive budding (B) in membranes of both cells and shedding of particles (LN) lacking nucleoids. Diffuse viroplasm (CD) consisting of virosomes, occasional virospheres (VS), and scattered virosome complexes (VX). X 75,000. (26).

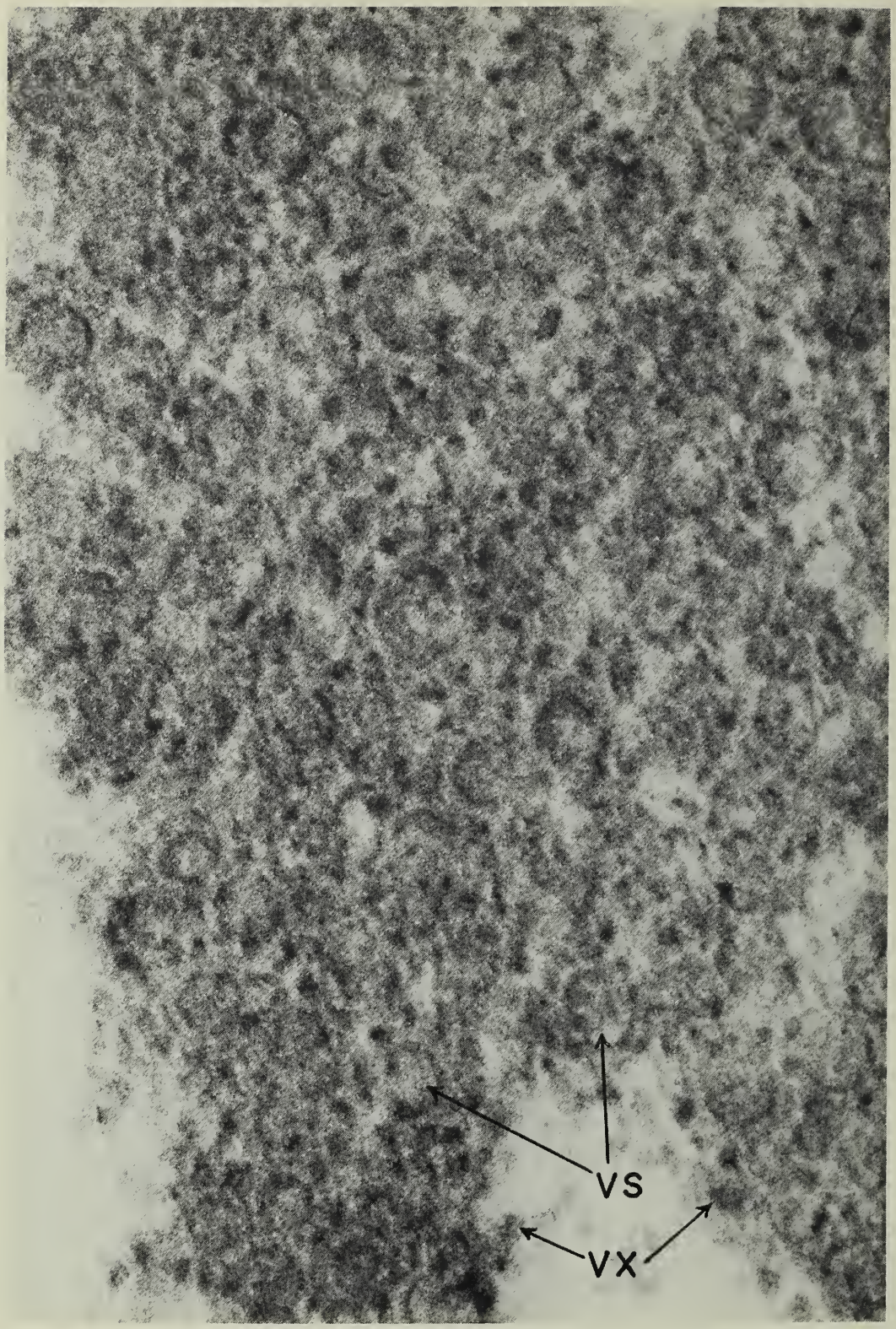

Figure 25.--Viroplasm of another cell of the same growth as the cells shown in figure 24. Virosomes (VX) are embedded in amorphous material of relatively high electron density. Numerous virospheres (VS) are bounded by irregular arrangements of spheroids or curved spindles of electron density like that of the virosomes. Central material of virospheres lacks uniformity of distribution. X 250,000. (26).

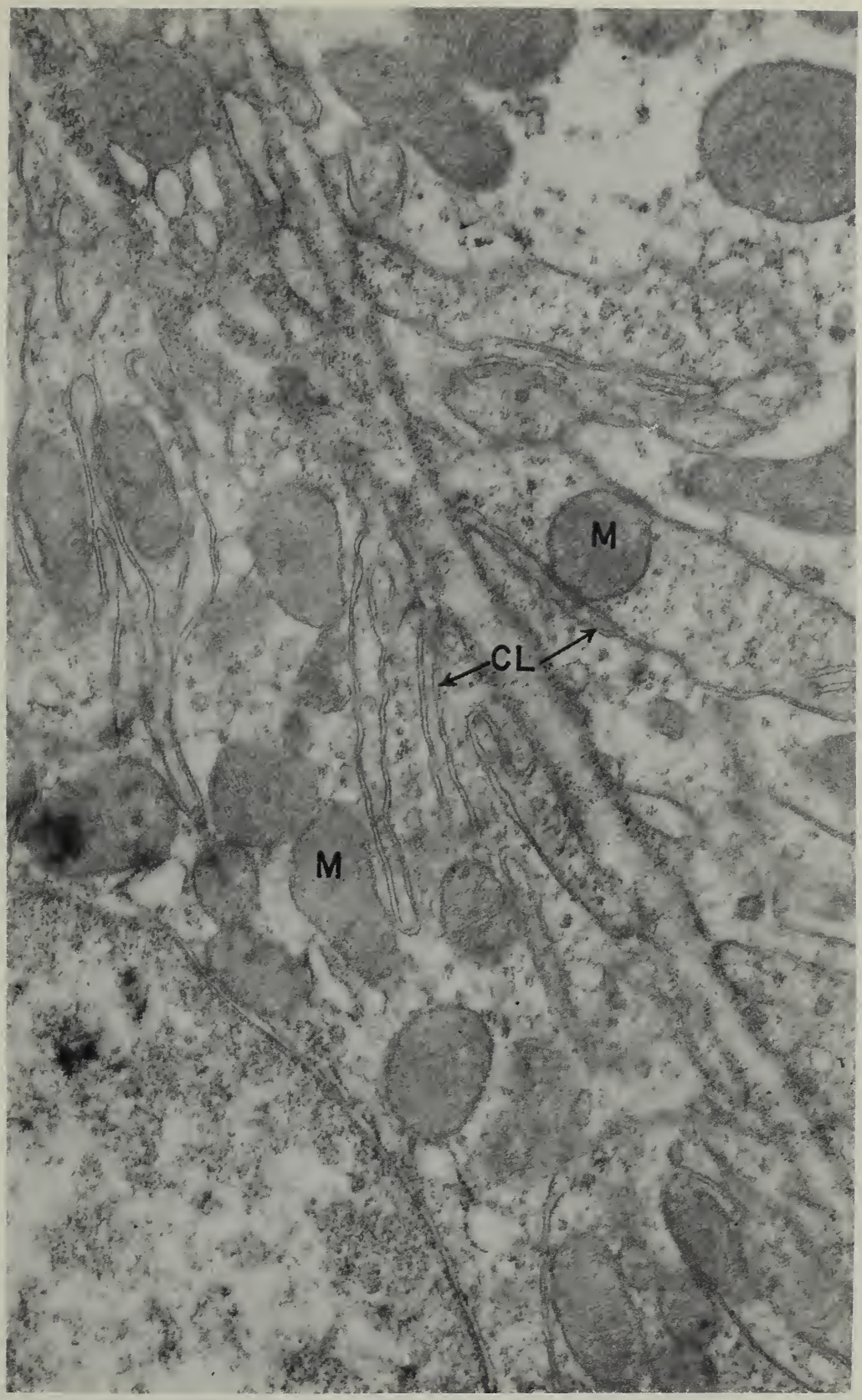

Figure 26.--Adjacent cells of proximal convoluted tubule of normal avian kidney. Mitochondria (M) of fine texture are in normal radial arrangement from cell base to apex. Note the characteristic inflection of cytoplasmic membranes to form thin canalicular loops (CL) that open externally through the basal cytoplasmic membrane. X 25,000. (26).

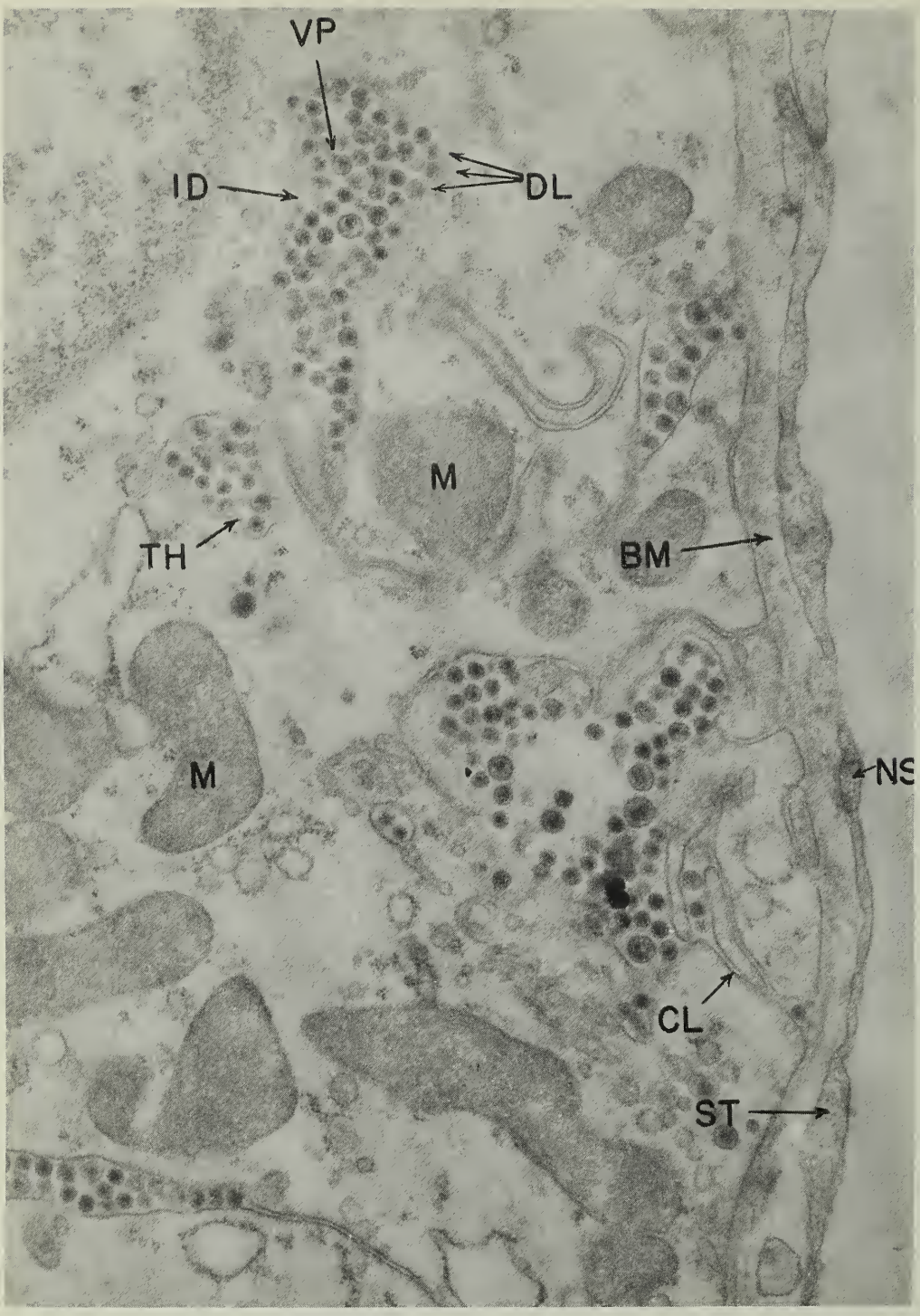

Figure 27.--Basal region of cell of distal convoluted tubule in nephroblastoma. Canaliculi (CL) are derived from cell membrane distended with virus particles (VP). Canalicular walls are single and thin (TH) or thickened and indistinct (ID). Denser membrane loci (DL) are suggestive of budding. A canaliculus at upper right is distended and open at cytoplasmic membrane. Cell cytoplasm is thin (fig. 26), mitochondria (M) are sparse, but cristae are fine and sharp; Canalicular system is poorly developed. Basement membrane (BM) is well developed, extratubular supporting stroma (ST) is delicate, and nuclei (NS) are spindle shaped. X 21,000. (26).

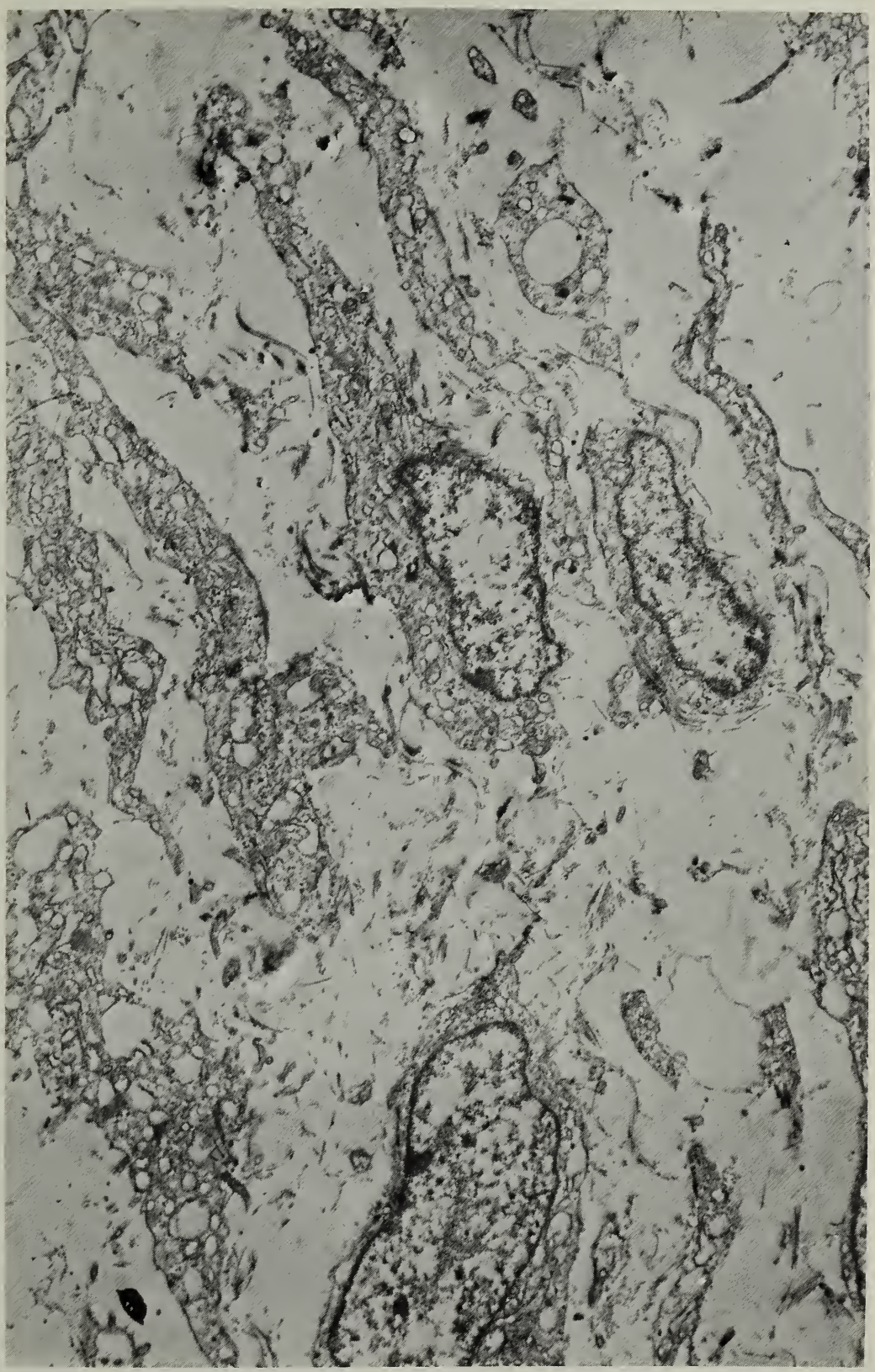

Figure 28.--Spindle-cell sarcoma with numerous cytoplasmic processes ramifying to contiguity with adjacent cells. Elements lie in amorphous, probably mucoid, material that contains relatively few single collagen fibrils which are arranged predominantly in laminated whorls or curved groups. Note the intensive and widely distributed process of collagen formation at cytoplasmic membranes. X 12,500. (26).

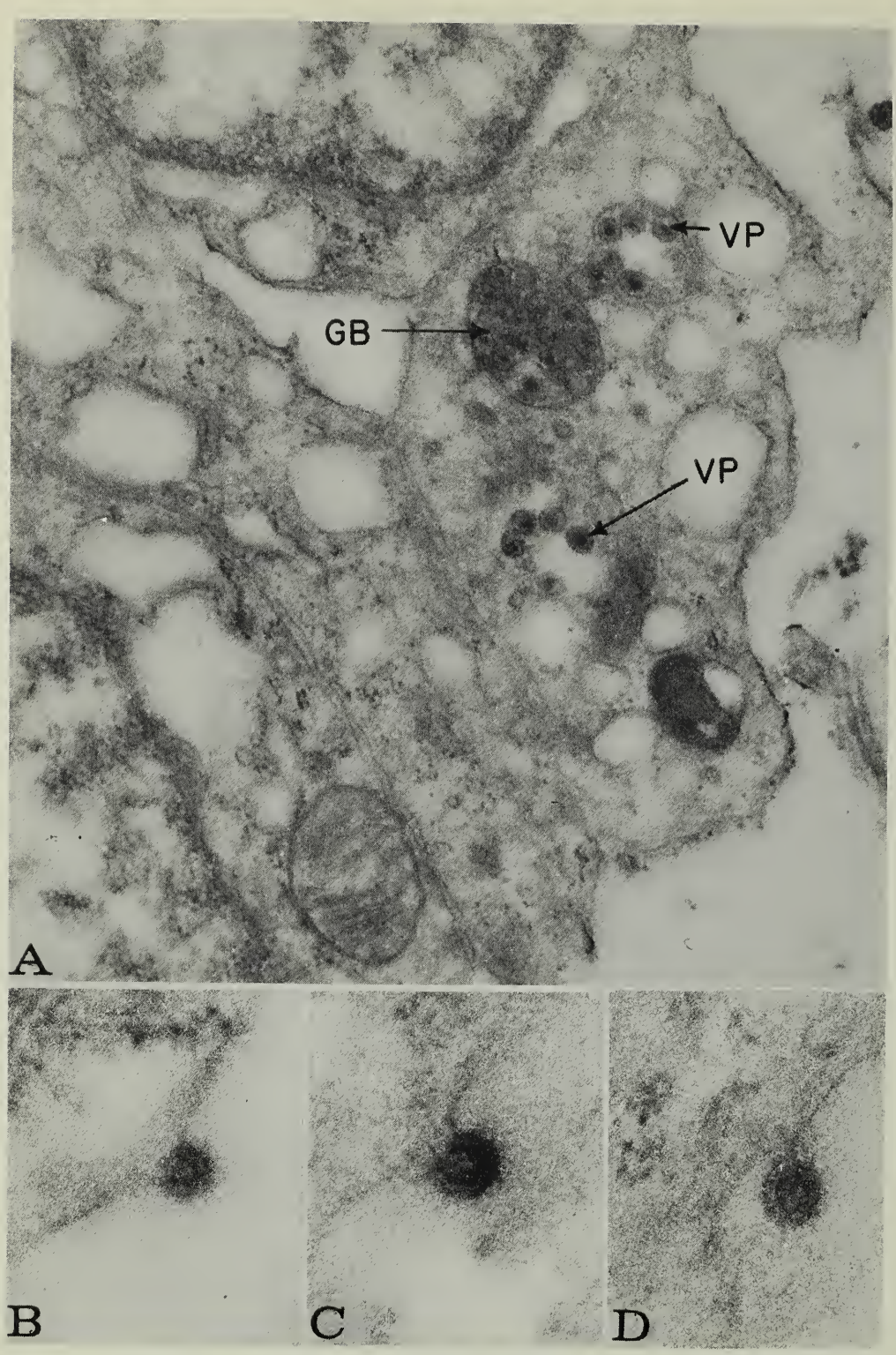

Figure 29.--A, Portions of four sarcoma cells that show virus particles (VP) in nearly clear cytoplasmic vacuoles. Gray body (GB), of unknown origin, is packed with particles of virus morphology. Ergastoplasm is poorly developed with large, irregular interlamellar spaces. Ribosomes are few in the cytoplasm and about the ergastoplasmic lamella. X 50,000. (26). B, C, and D, Progressive stages of virus-particle formation by budding from the plasma membranes of separate sarcoma cells in two tumors. X 100,000. (26).

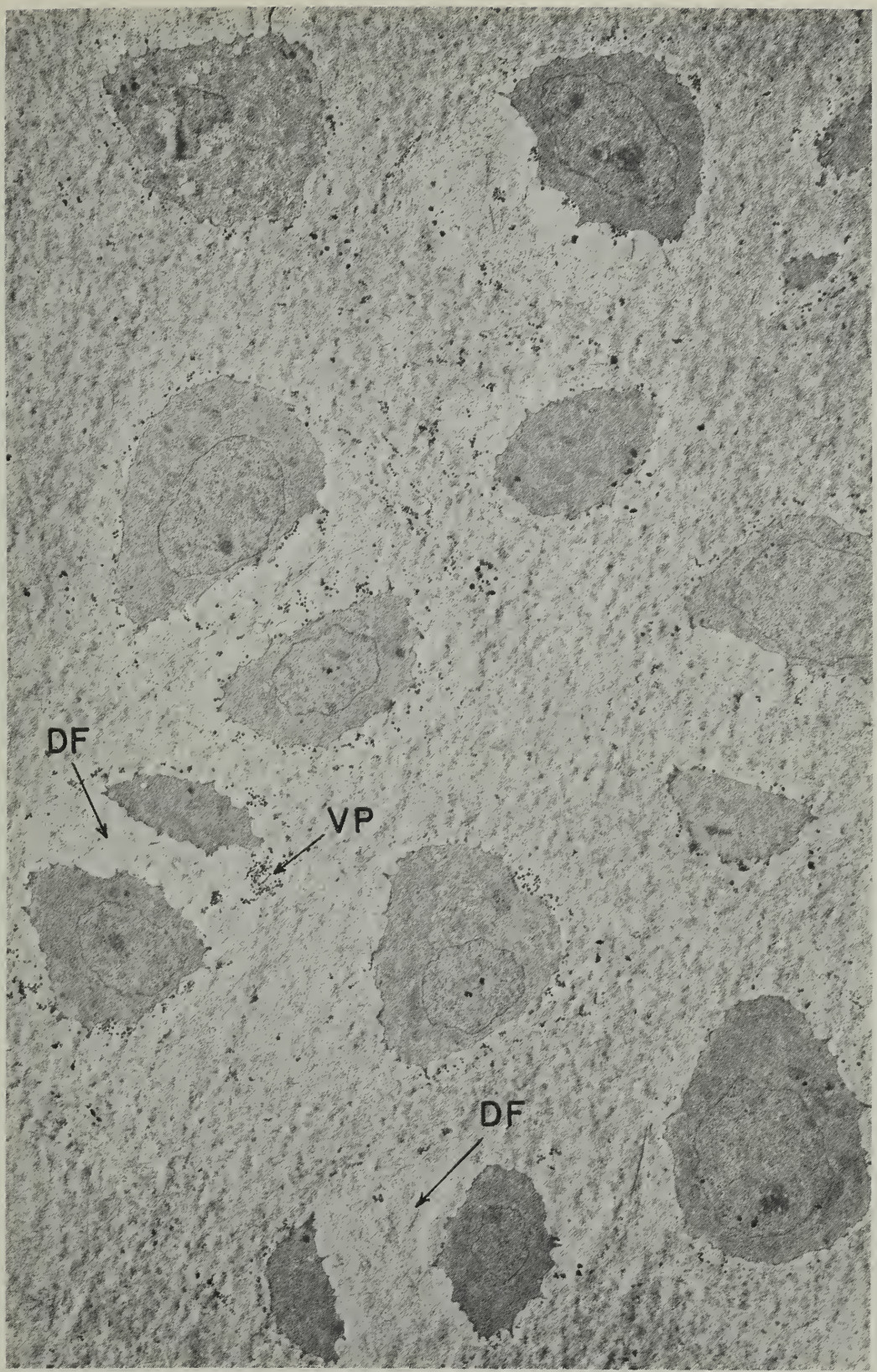

Figure 30.--Cartilage with individual and paired chondrocytes after division (DF) in a matrix replete with cartilage fibrils. Numerous virus particles (VP) are trapped in matrix about cells. A few particles between cells have divided. X 3,600. (26).

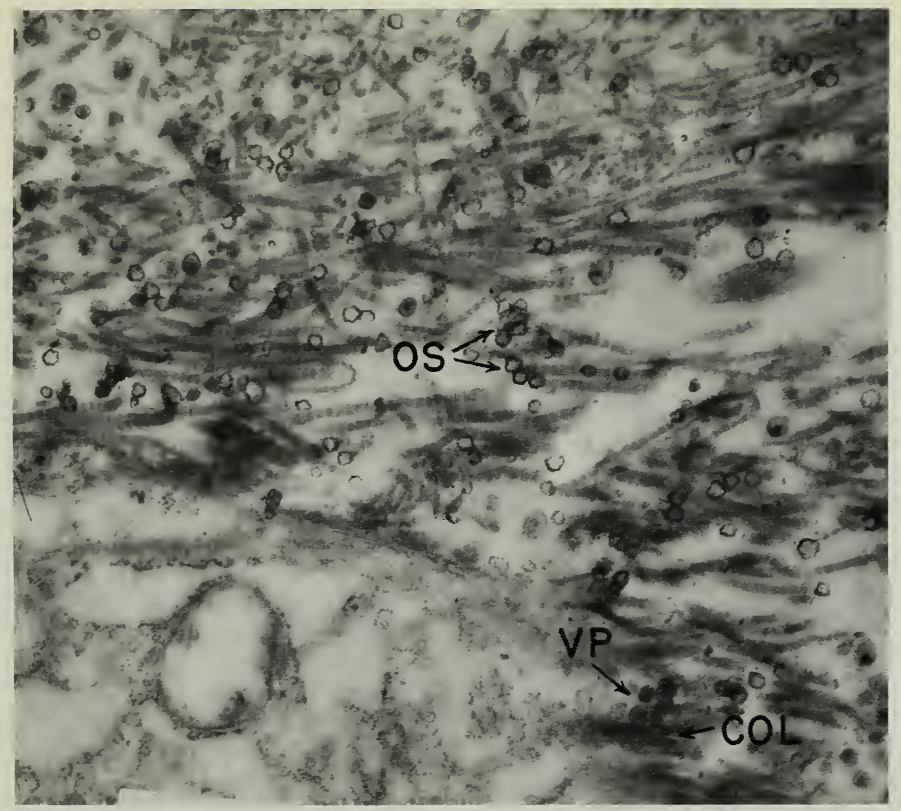

Figure 31.--Edge of cartilage deposit. Upper cell (CB) has the morphology of prechondrocyte with highly developed, intricate ergastoplasm. Intensive process of collagen-fiber formation is shown at upper cell membrane (COL), and the elaboration of collagen and cartilage fibers are shown at right. Cartilage fibril deposit (CAF) is at lower membrane. Large numbers of virus particles (VP) elaborated at membrane are in meshes of fibers and fibrils. Young chondrocyte (CC) of essentially characteristic morpnology is just beneath surface of cartilage and is in the process of virus-particle formation and fibril elaboration. X 9,500. (26).

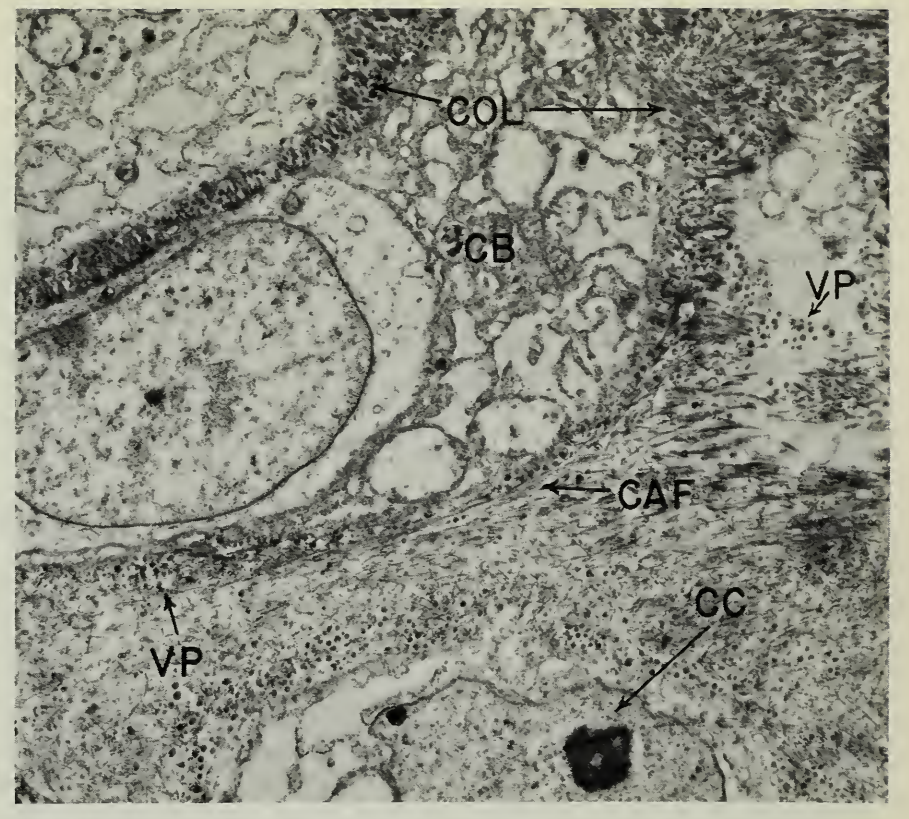

Figure 32.--Massive collagen elaboration near site of the cells shown in figure 31. At lower right (COL), fibers extend as a fan of fibrils into the cell cytoplasm. Virus particles (VP) lie in the mesh of fibers. The open spheres (OS) are approximately the size of virus particles, and some contain nucloeoids. These may represent aberrantly formed particles with membranes containing collagen constituents incorporated in the process of budding from plasma membrane engated in collagen formation. X 22,000 (26).

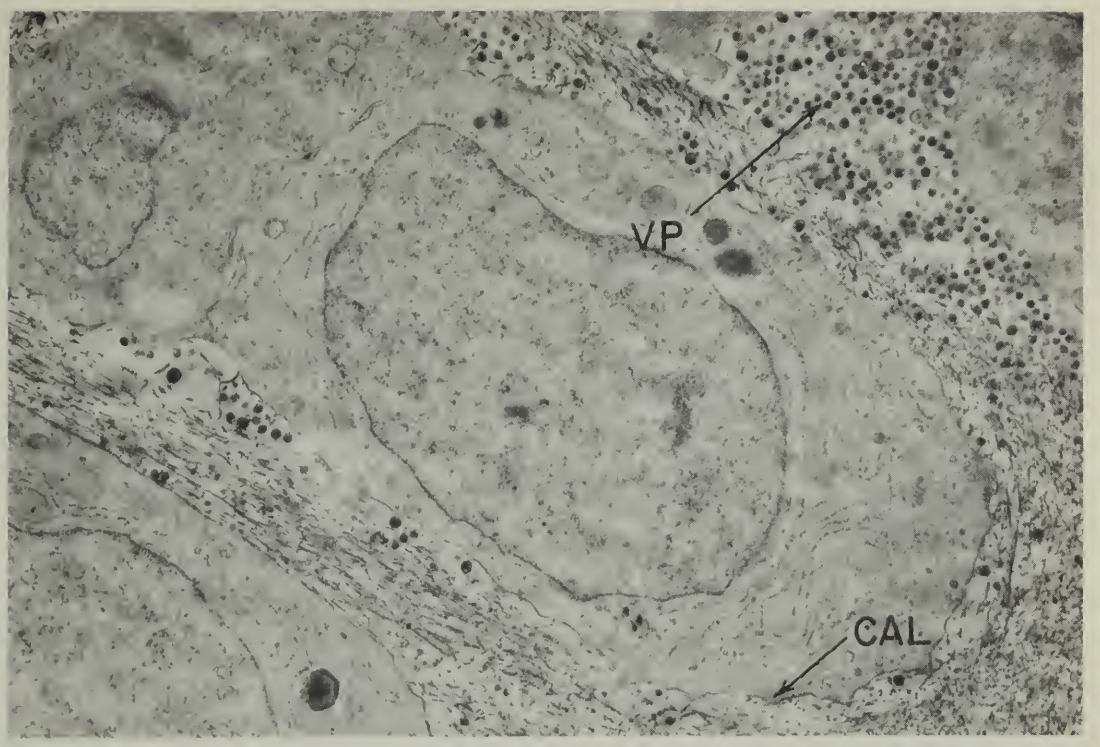

Figure 33.--Mature chondrocyte, relatively normal in morphology, with surrounding virus particles (VP) and fibrils (CAL) splitting from the plasma membrane. X 15,000. (26).

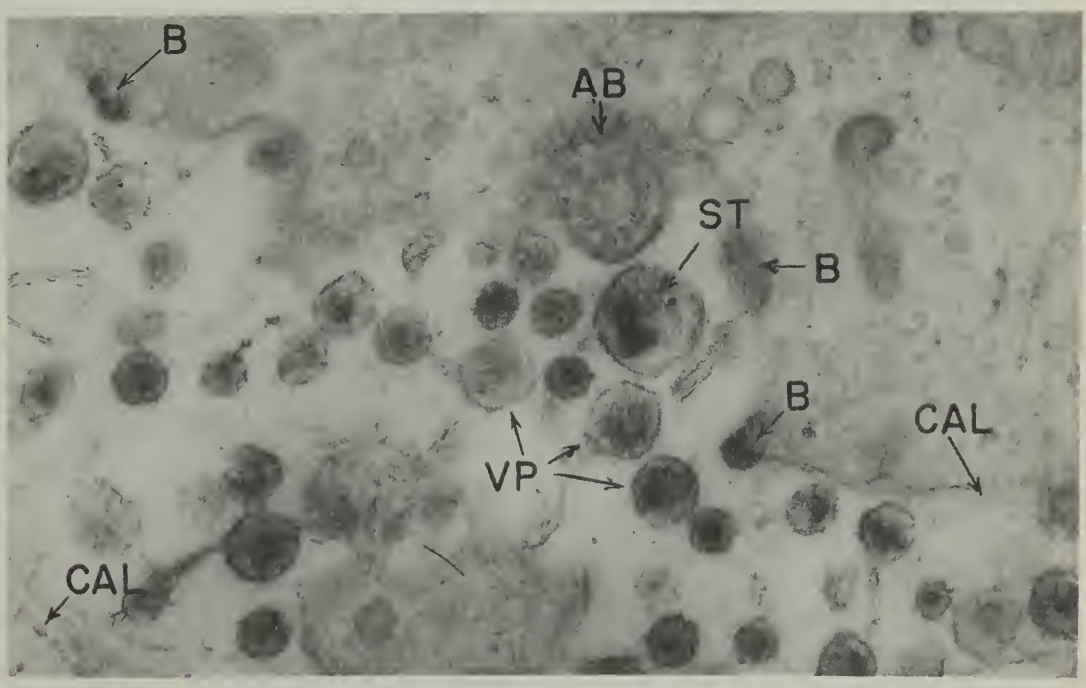

Figure 34.--Higher magnification of chondrocyte, like that in figure 34, showing typical and usual morphology of cartilage fibrils (CAL) and virus particles (VP). Several virus buds (B) are seen in plasma membrane. Several free virus particles show threadlike striae (ST) that resemble pieces of cartilage fibrils like those (CAL) splitting from the plasma membrane and others free in the cartilage matrix. The bulbous protuberance (AB) from the cell membrane was not identified. X 55,000. (26).

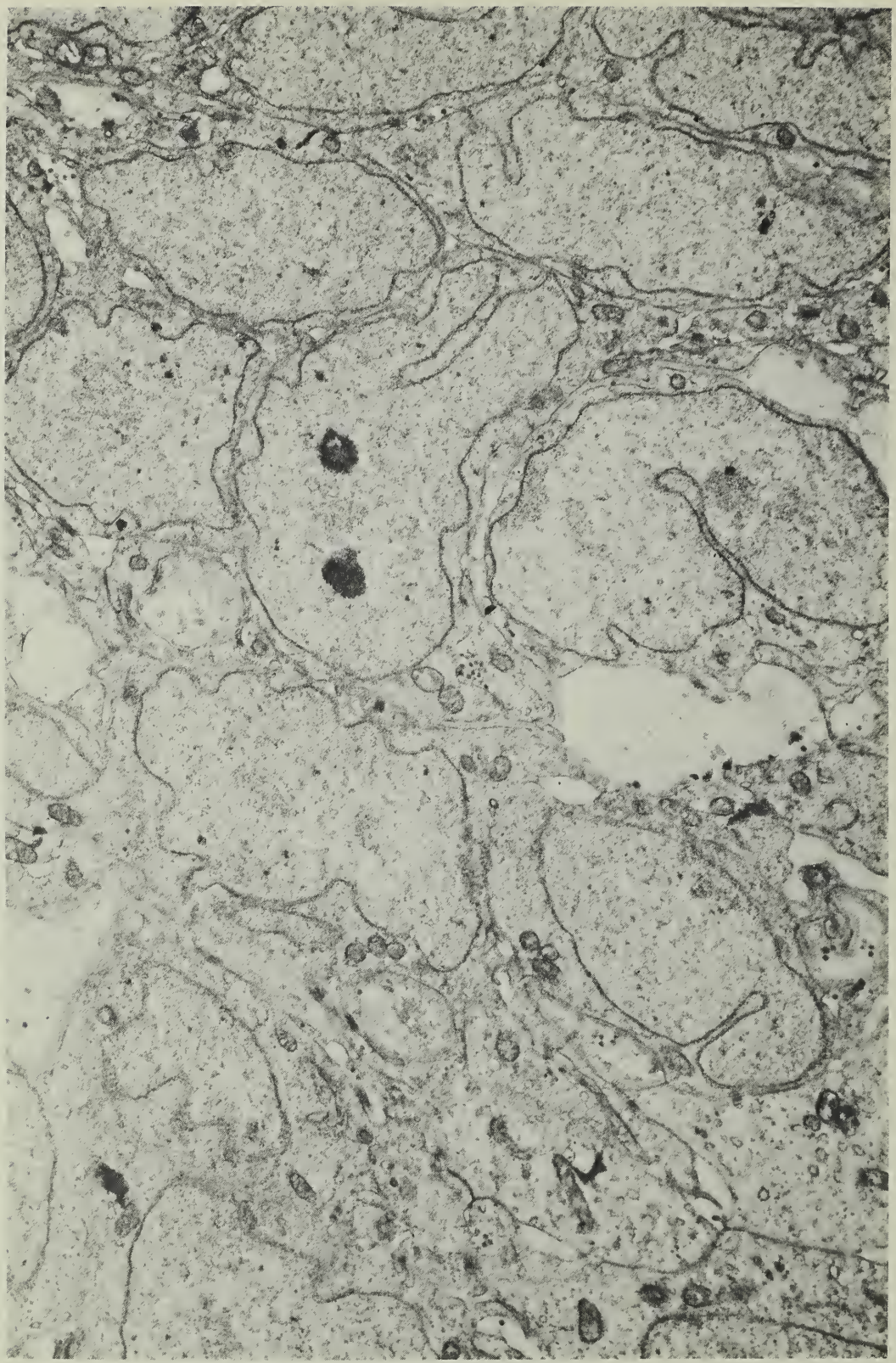

Figure 35.--Incompletely differentiated stromal cells like those (UM) in figures 11 and 12. These cells exhibit some morphologic features that resemble those of undifferentiated mesenchyme in the cortex of the normal embryo kidney (fig. 36). X 12,000. (26).

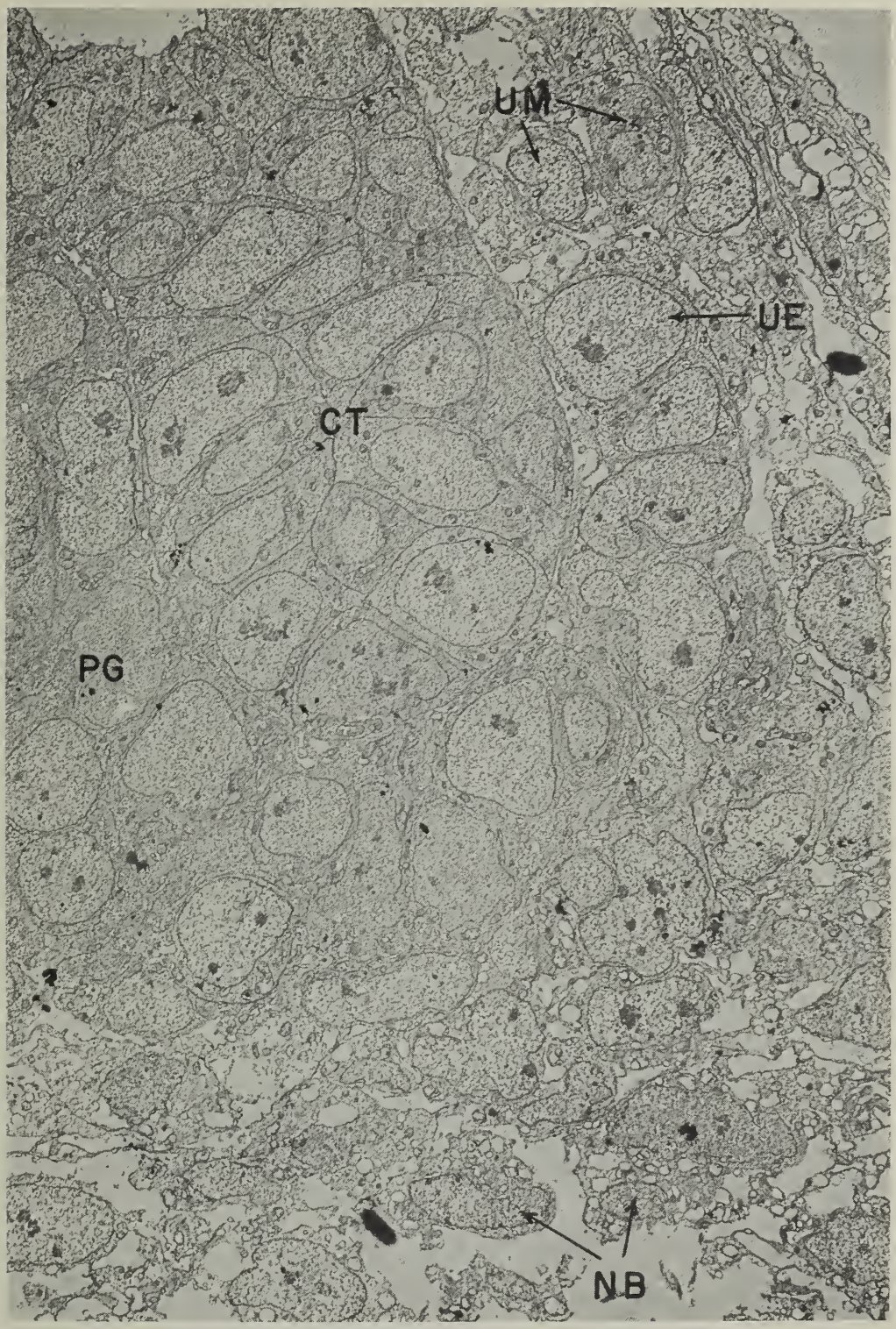

Figure 36.--Horn of ingrowing collecting tubule (CT) with adjacent inverted, comma-shaped primitive nephrogenic mass (PG) just beneath renal capsule of 13-day chick embryo. Mesenchymal cells (NB) exhibit morphology of renal-cortical nephroblastema before ingrowth of collecting tubules. Cells (UM) near tail of comma suggest transitional differentiation toward epithelium, and neighboring elements (UE) appear as incompletely differentiated primitive tubule epithelium. X 3,500. (26).

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